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**Moulds and *Staphylococcus aureus* enterotoxins are relevant allergens to affect type2 inflammation and clinical outcomes in CRS patients**

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**A short running title:** Alt/SEs are relevant allergens to develop UADs

**Word table and figure counts:** 241 and 2932 words, 4 tables, and 1 figure, respectively

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Y.K.: established the conception of the whole study, and contributed to the performance of diagnostic tests, the collection of data, the recruitment of patients, disease diagnosis and management, the acquisition and interpretation of data, and drafting the manuscript. K.F., R.K., and N.T. contributed to the performance of diagnostic tests, the collection of data, and the acquisition and interpretation of data. J.Y. contributed to the acquisition

and interpretation of data, as well as proofreading of the manuscript in English. H.N., S.F., T.U., T.T., H.O., K.M., Y.I., and T.O. contributed to the diagnostic tests, the collection of data, and management of patients. J.O. and K.I. carried out the measurement of periostin. A.M. made specimens and assessed the infiltration of eosinophils in upper airway tissues. Y.O. contributed to assess the radiological severity of CRS. M.T. contributed to the recruitment of patients, disease diagnosis and management, and revision of the manuscript. M.S. and A.N. contributed to the recruitment of patients, disease diagnosis and management, interpretation of data, and revision of the manuscript.

**Conflict of Interest Statement:**

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**Abbreviations:**

AQLQ: Asthma Quality of Life Questionnaire, CRS: chronic rhinosinusitis, CRSwNP: chronic rhinosinusitis with nasal polyps ESS: endoscopic sinus surgery, FeNO: fractional nitric oxide, LMS: Lund–Mackay score, HPF: high power field, Moulds/SEs: *Alternaria, Aspergillus* and/or *Staphylococcus aureus* enterotoxins, NPs: nasal polyps,

QoL: quality of life, SEs: *Staphylococcus aureus* enterotoxins, SNOT-22: Sino-nasal Outcome Test-22

**Abstract:**

**Background:** Sensitization to moulds and *Staphylococcus aureus* enterotoxins (SEs) is associated with the pathophysiology of both asthma and chronic rhinosinusitis (CRS). The purpose of this study was to clarify the contribution of these allergens sensitization on type2 inflammation in blood, nose, and lower airways, and clinical outcomes in CRS patients.

**Methods:** We prospectively enrolled 56 CRS patients who underwent endoscopic sinus surgery (ESS) (20 with comorbid asthma), and 28 healthy controls between October 2015 and December 2017. CRS patients were followed 12 months after surgery. Type2 inflammation-related biomarkers were analyzed using blood, resected tissue samples, and sputum. Ten allergens including *Alternaria*, *Aspergillus,* and SEs were measured. Type2 inflammation-related biomarkers and clinical outcomes were compared in the stratification with the presence or absence of allergen sensitization.

**Results:** Sensitization rate to moulds and SEs in asthmatic patients was increased when changing the cut-off value of specific IgE titer from 0.35 UA/mL to 0.10 UA/mL (1.7 and 4.5 folds, respectively). Moulds and SEs affected the prevalence of asthma and eosinophilic CRS by interacting with each other. All type2-related biomarkers but eosinophils in sinus tissue were significantly higher in patients with moulds or SEs

(moulds/SEs) sensitization (≥0.10 UA/mL) (n = 19) than in those without (n = 37) and healthy subjects (all p <0.05). Meanwhile, moulds/SEs sensitization did not affect longitudinal changes in clinical outcomes after ESS. Changes in serum moulds/SEs-IgE levels by ESS remained unclear.

**Conclusion:** Moulds/SEs sensitization (≥0.10 UA/mL) may affect the development of type2 inflammation and clinical outcomes in CRS patients.

**Take home message:**

*Alternaria*, *Aspergillus*, and *S. aureus* enterotoxins are important allergens to affect type2 inflammation and clinical outcomes in CRS patients. Sensitization to moulds/SEs (≥0.10 UA/mL) would be meaningful in the pathophysiology of CRS.

**Keywords**: atopy, moulds, *Staphylococcus aureus* enterotoxins, asthma, (asthma with) chronic rhinosinusitis

**Introduction:**

Sensitization to allergens is a significant risk factor for adult asthma onset in general population[1, 2]. It is also prevalent in patients with chronic rhinosinusitis (CRS), which ranges from 50 to 84%[3]. Generally, we consider patients as having sensitization to allergens when one or more specific IgE tiers against allergens show ≥0.35 UA/mL[4]. Meanwhile, several studies indicate the association of threshold of ≥0.10 UA/mL in serum specific IgE titers including *Staphylococcus aureus* (SA) enterotoxins (SEs) and *Asperguillus* with onset, severity, and poor control of asthma[5-9]. However, the contribution of lower threshold (≥0.10 UA/mL) of serum specific IgE titers against allergens on type2 inflammation and clinical outcomes in allergic diseases remains poorly known.

Long-term colonization of moulds and SA to the nose and lower airways play a role in their sensitization systemically or locally[10, 11]. Levels of IgE in the nose and lower airways is associated with local eosinophilic inflammation[12, 13]. *Alternaria* and *Aspergillus* are well-known fungal allergens to associate with severe asthma[14, 15], along with SA[6-8]. These suggest that environmental microorganism-related allergens may be strong activators of type2 inflammation throughout airways and contribute to worse clinical outcomes in CRS patients as compared to other common allergens.

The objective of this study was to explore different cut-off levels of sensitization including moulds and SEs in relation to type 2 inflammation-related biomarkers in blood, nose, and lower airways, and the clinical relevance in patients with CRS. Furthermore, we assessed if moulds and SEs sensitization had any effects on the clinical and inflammatory outcomes 12 months after endoscopic sinus surgery (ESS).

**Methods:**

This study is a post hoc analysis of our previous study which evaluated the pathophysiological link between upper and lower airways in CRS patients[16]. We prospectively enrolled 56 CRS patients who agreed to ESS (20 with and 36 without comorbid asthma), and 28 healthy controls between October 2015 and December 2017. The diagnosis of asthma and CRS was made according to guidelines[17, 18] . These were described in the previous report along with inclusion and exclusion criteria of the study[16]. We excluded current smokers in this study. We confined patients to those who underwent ESS in this study because tissue eosinophilia in sinus, particularly in NPs, is associated with the presence of asthma[19, 20]. We also suggested that upper airway tissue eosinophilia is pathophysiologically linked to type2 lower airway inflammation. Indeed, we have demonstrated the association of tissue eosinophilia in sinus and NPs with type2 lower airway inflammation in our previous study[16]. This study was approved by the Ethics Committee of our hospital (1165) and was registered on the UMIN Clinical Trials Registry (Registry ID UMIN000018672). Written informed consent was obtained from all participants.

**Measurements**

All participants underwent spirometry, fractional nitric oxide (FeNO)

measurement (oral expiratory flow rate with 50 ml/s), olfactory function test as assessed by the Open Essence method (ranging from 0 to 12, and lower scores showing impaired olfaction)[21] and sputum induction, and answered the Sinonasal Outcome Test-22 [SNOT-22; ranging from 0 to 110, and higher scores showing worse sinonasal-related quality of life (QoL)][22] before ESS as did healthy subjects. One patient was not able to measure FeNO levels at enrollment due to apparatus failure. Additionally, blood and tissue samples collection and sinus CT scan were performed only in CRS patients. Serum type2 inflammation-related biomarkers were also determined in 20 healthy subjects using stored serum samples. Inflamed sinus tissue samples and NPs were taken from 54 and 38 patients under general anesthesia by an otorhinolaryngology specialist (M.S.), respectively. Eosinophil counts and periostin levels were measured using blood and sputum samples. The number of eosinophils in sinus and NPs (high power field, 400×) and the Lund–Mackay score (LMS; ranging from 0 to 24, and higher scores showing severe CRS) of sinus CT were determined by a pathologist (A.M.) and a radiologist (Y.O.) under a blinded manner, respectively. We defined eosinophilic CRS when eosinophils in sinus or NP tissue showed ≥70 HPF[19]. We re-evaluated all measurements aside from tissue collection were 12 months after ESS in CRS patients. Patients with asthma also completed the Asthma Quality of Life Questionnaire[23]

(AQLQ; comprising 4 domains, ranges 0 to 7, and lower scores showing worse asthma-related QoL) at both before and 12 months after ESS. We obtained the permission to use the SNOT-22 and the AQLQ for this study from Professor Jay Piccirillo, Washington University, USA, and Professor Elizabeth Juniper, McMaster University, Canada, respectively. Furthermore, an otorhinolaryngologist (M.S.) and asthma specialists (Y.K., M.T. and A.N.) assessed the recurrence of NPs and new asthma onset for 12 months following ESS, respectively. Detailed information of these measurements was described in previous report[16].

**Allergens measurement**

We determined serum total Immunoglobulin E (IgE) and specific IgE antibody against ten allergens; house dust mite, cat, dog dander, Japanese cedar pollen, mixed Gramineae pollens (orchard grass, sweet vernal grass, Bermuda grass, Timothy grass and reeds), mixed weed pollens (ragweed, mugwort, goldenrod, dandelion and oxeye daisy), *Alternaria alternata*, *Aspergillus fumigatus*, and *Staphylococcus aureus* enterotoxin A and B (SEA and SEB, respectively) (ImmunoCAP®; Phadia K.K., Tokyo, Japan). Detection limit for each specific IgE titer was 0.10 UA/mL. Table 1 shows the proportion of each sensitized allergen in CRS patients when the cut off value of serum specific IgE titer was set at 0.10 UA/mL or 0.35 UA/mL. Sensitization rate to moulds

and SEs in asthmatic patients was increased when changing the cut-off value of specific IgE from 0.35 UA/mL to 0.10 UA/mL (1.7 and 4.5 folds, respectively) (Table 1). Meanwhile, sensitization to other allergens such as mite and pollens was similar between two cutoff values of specific IgE titers. When a cut-off value was set at 0.10 UA/mL, there were significant differences of sensitization to moulds or SEs between CRS patients with and without asthma (Table 1). This suggests that sensitization to moulds or SEs (≥0.10 UA/mL) would be meaningful in the pathophysiology of both asthma and CRS. Thus, we considered patients as having sensitization to moulds or SEs if they showed serum specific IgE titer of ≥0.10 UA/mL in the present study. On the other hand, we considered participants as having other conventional allergens sensitization if they showed one or more specific IgE titers against remaining allergens of ≥0.35 UA/mL.

To compare the prevalence of moulds and SEs sensitization between CRS patients and healthy subjects, we also measured serum total IgE levels and specific IgE titers against *Alternaria*, *Aspergillus*, and SEs in healthy subjects. Sensitization rate to either moulds or SEs were similar between all CRS patients and healthy subjects (Table1).

**Statistics**

Data were analysed using JMP 14 Start Statics (SAS Institute Inc., Cary, NC,

USA) and presented as median ($25^{th}$ percentiles, $75^{th}$ percentiles). We evaluated the interactive effect between moulds and SEs sensitization for clinical outcomes in CRS patients using analysis of co-variance. If a p value showed <0.05, they affected the development of clinical outcomes by interacting each other. We also evaluate the interactive effect of smoking or other conventional allergens with moulds or SEs sensitization for clinical outcomes. We stratified patients according to the presence or absence of sensitization to allergens (allergen+ and allergen- groups). We adopted Kruskal-Wallis test followed by Steel-Dwass analysis or Chi square test when comparing indices among patients with and without allergen sensitization and healthy subjects. We also applied Wilcoxon rank sum test or Fisher exact test when comparing between allergen+ and allergen- groups. We analyzed changes in indices with ESS using Wilcoxon single rank test. A p value $\leq 0.05$ was considered significant when $\alpha$ error was set at 5 %.

**Results:**

Characteristics of participants are presented in Table 2. The proportion of female participants was lower in patients with moulds or SEs sensitization than in healthy subjects. Smoking history was less frequent in healthy subjects than CRS patients, however, it was unrelated to sensitization to either moulds or SEs (≥0.10 UA/mL) in CRS patients. There were no obvious differences between age and body mass index among the three groups. When confined to patients with CRS, the prevalence of asthma and-sensitization to other allergens were more frequent in patients with moulds or SEs sensitization than in those without (Table 2). Meanwhile, other general indices such as previous history of ESS and diseases duration were similar between the two groups (Table 2).

**Interactive effect between moulds and SEs for clinical outcomes in CRS patients.**

We evaluated whether moulds and SEs have the interactive effect for the development of clinical outcome in CRS patients. Moulds and SEs significantly increased serum periostin levels and sputum eosinophil counts and affected the prevalence of asthma and eosinophilic CRS by interacting with each other (Table E1). This result suggests that moulds and SEs induce type2 inflammation systemically and locally by interacting with each other. Indeed, 10 patients (53%) were sensitized to both

moulds and SEs. Therefore, we categorized these allergens into the same group (moulds/SEs) to evaluate their relevance to clinical outcomes in CRS patients. However, other conventional allergens did not have interactive effect with moulds/SEs allergens for prevalence of asthma and eosinophilic CRS (data not shown).

**The impact of sensitization to moulds/SEs** (≥0.10 UA/mL) **on clinical outcomes.**

We evaluated the impact of sensitization to moulds/SEs (≥0.10 UA/mL) on clinical outcome (Table 3). All type2 inflammation-related biomarkers aside from eosinophil counts in sinus tissue were significantly higher in moulds/SEs+ group than in moulds/SEs- group (Table 3). These significances were not observed except for serum total IgE levels upon setting a cut-off value at 0.35 UA/mL (Table E1). Type2 lower airways inflammation in moulds/SEs+ group was also more predominant than in healthy subjects, while its difference between moulds/SEs- group and healthy subjects was only seen in sputum periostin levels (Table 3). Conventional allergens have interactive effect with moulds/SEs allergens for the increase in serum periostin levels and sputum eosinophil counts, whereas smoking did not have any interactive effect with moulds/SEs sensitization for the development of clinical outcomes in CRS (data not shown).

Sensitization to moulds/SEs (≥0.10 UA/mL) did not affect olfaction, radiological CRS severity, or sinonasal- and asthma-related QoL in CRS patients (Table 3).

**The impact of sensitization to moulds/SEs (≥0.10 UA/mL) on the longitudinal clinical outcomes**

We also evaluated the association of sensitization to moulds/SEs (≥0.10 UA/mL) with changes in biomarkers, radiological CRS severity, olfaction and QoL by ESS intervention (Table 4). We traced 48 CRS patients for 12 months after ESS (15 with having moulds/SEs (≥0.10 UA/mL) sensitization and 33 without). Among 48 patients 15 had moulds or SEs sensitization at enrolment (n = 11 for both, respectively). Although serum moulds-IgE levels turned negative in only one patient without asthma, none sensitized one year after ESS. On the other hand, serum SEs-IgE levels turned negative in four patients (two with asthma and two without asthma) whereas six (two with asthma and four without asthma) newly sensitized after surgical intervention. Twenty patients had moulds or SEs sensitization one year after ESS. We could not find any significant difference between patients whose serum moulds/SEs-IgE levels were changed by ESS.

ESS could improve radiological CRS severity, olfaction and sinonasal- and asthma-related QoL in CRS patients (Figure 1, Table 4). Moreover, levels of sputum periostin also significantly declined with ESS intervention (Figure 1, Table 4). When patients were divided into the presence or absence of sensitization to moulds/SEs (≥0.10

UA/mL), however, changes in biomarkers, radiological CRS severity and QoL with ESS intervention were similar between the two groups (Table 4). The proportion of the recurrence of NPs or new asthma onset for 12 months following ESS were not also related to moulds/SEs sensitization (≥0.10 UA/mL) at enrolment.

## Discussion

To the best of our knowledge, this is the first study to demonstrate that levels of moulds/SEs-IgE of ≥0.10 UA/mL rather than ≥0.35 UA/mL may be more useful biomakers that reflects systemic and local type2 inflammation and asthma prevalence in CRS patients. They contributed to the development of clinical outcomes in CRS patients by interacting with each other. Meanwhile, either mould or SEs sensitization (≥0.10 UA/mL) did not affect changes in clinical outcomes and type2 inflammation-related biomarkers with ESS.

Specific IgE titers are measured using fluorescence enzyme immunoassay. A cut-off value of this assay was set at 0.35 UA/mL because of strong correlation with radioallergosorbent test in 1990[4]. Meanwhile, detection limit of specific IgE titers have improved from 0.35 UA/mL to 0.10 UA/mL for the last two decades. We have demonstrated the importance of a lower cut-off value of moulds/SEs-IgE (≥0.10 UA/mL) on the development of type2-predominant inflammation in nose and lower airways. Sensitization rate to moulds and SEs in asthmatic patients rose from 10-30% to 45-50% when the cut-off value was set at 0.10 UA/mL than at 0.35 UA/mL (Table 1). However, the change of a cut-off value from 0.35 UA/mL to 0.10 UA/mL hardly affected the frequency of patients who have sensitization to other allergens such as

HDM, cat, and pollens (Table 1). Moulds and SEs might induce type2-predominant airway inflammation by lesser amount than other conventional allergens. Recent studies have reported significant association of a SEs sensitization determined by 0.10 UA/mL with the prevalence[2] and severity of asthma[7]. Thus, a lower cut-off value of 0.10 UA/mL in moulds and SEs would be meaningful in the pathophysiology of allergic diseases such as asthma and CRS. Moulds and SEs may be common allergens in patients with asthma as well as other conventional allergens.

*Alternaria* and *Aspergillus* are classified into moulds, whereas SA are bacteria. Both moulds and SA are particularly colonized in NP of CRS patients[24-26]. We found that moulds and SEs have interactive effect for the development of clinical outcomes and type2 inflammation in CRS patients, however, different mechanisms are associated with the development of type2-predominant inflammation. Although enterotoxins produced by SA are well-known inducers of type2-related cytokines and eosinophilic inflammation in both nose and lower airways by acting as superantigens[27, 28], SA can directly induce interleukin-33 and thymic stromal lymphoprotein production via airway epithelium through binding to Toll-like receptor 2[29]. *Alternaria alternata* and *Aspergillus fumigatus* share very similar epitope[30]. This suggests that their sensitizations may elicit similar immune response. Protease produced by *Alternaria* and

*Aspergillus* can evoke release of type2-related cytokines[31], airway hyperresponsiveness[32], and activation of eosinophils[33]. Furthermore, humoral immune response to moulds is also thought to be associated with increased production of type2-related cytokines[34]. Lesser amount of sensitization to moulds and SEs may be related to induce type2 inflammatory cascade in nose and lower airways as stated above.

Airways are anatomically continuous from the nose to lower airways. Sino/naso-bronchial reflex is thought to be associated with the development of both upper and lower airway inflammation. Inhalation of allergens through the nose and lower airways can induce type2 inflammation of lower airways and nose, respectively[35, 36]. Nasal colonization of SA is also related to the development of eosinophilic NPs (i.e. eosinophil cationic protein production)[25] and asthma prevalence[37]. These evidence support the presence of the interaction between upper and lower airway inflammation by neuronal reflex. However, all type2 inflammation-related biomarkers of lower airways except for sputum periostin did not decline by ESS. Furthermore, the presence of moulds/SEs did not affect either improvement of clinical outcomes or changes in type2 inflammation-related biomarkers one year after ESS. Although we did not evaluate levels of moulds/SEs-IgE in NPs, it

remains unclear whether ESS provides changes in levels of serum moulds/SEs-IgE. These results indicate that systemic allergen sensitization induces type2 inflammation in nose and lower airways separately. Neuronal reflex through local allergen sensitization may not be major cascade in the development of type2 airway inflammation throughout airways. Indeed, treatment of CRS does not always improve asthma outcomes[3]. Meanwhile, most of type2 inflammation-related biomarkers declined numerically by ESS in moulds/SEs+ group. Sample size may not be sufficient to clarify the impact of moulds/SEs on longitudinal changes in clinical outcomes and type2 inflammation-related biomarkers in CRS patients who underwent ESS. Further larger cohort studies are necessary to elucidate how moulds/SEs sensitization affect clinical symptoms and inflammation of nose and lower airways after ESS.

There are some limitations in the present study. First, we did not evaluate local levels of IgE against moulds/SEs either sputum or sinus sample due to the lack of storage samples. Therefore, the association between lower threshold (≥0.10 UA/mL) of serum IgE titers and local sensitization to allergens remains to be elucidated. However, the present study provides clinicians with clinical importance of sensitization to moulds/SEs (≥0.10 UA/mL) as indicators of the presence of type2 inflammation and comorbid asthma in CRS patients. Both otorhinolaryngology and respirology specialists

can notice comorbid asthma or eosinophilic CRS by evaluating specific IgE against moulds/SEs. Second, our cohort enriched more severe CRS patients who were required to ESS. Therefore, the present findings could not adapt CRS patients who are mild/moderate or responsive to treatment are not captured. We need to investigate the difference of the impact of moulds/SEs sensitization (≥0.10 UA/mL) between severe and non-severe CRS patients on type2 inflammation-related biomarkers and clinical outcomes in the future study. Last, follow-up of patients was finished 12 months after ESS. Therefore, the association of sensitization to moulds/SEs (≥0.10 UA/mL) with long-term prognosis of ESS for asthma and CRS remains unclear. Further longitudinal studies are necessary to clarify clinical implication of sensitization to moulds/SEs determined by ≥0.10 UA/mL on the pathophysiology of asthma and sinusitis.

In conclusion, moulds/SEs are important allergens in developing comorbid asthma and eosinophilic CRS and inducing type2 inflammation in CRS patients. A lower threshold value of these allergens (≥0.10 UA/mL) would be meaningful to evaluate type2 inflammation and clinical outcomes in CRS patients. To further clarify the clinical implication of moulds/SEs sensitization determined by ≥0.10 UA/mL on the pathophysiology of asthma and CRS, subsequent studies are warranted.

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**Figure legend:**

**Figure 1. The efficacy of endoscopic sinus surgery in CRS patients**

Box and whisker plots show the change in the LMS (A), the SNOT-22 score (B), olfactory score (C), the AQLQ score (D), and levels of sputum periostin (E) with endoscopic sinus surgery. The horizontal line in the box interior shows the median values of indices. The length of the box represents the distance between the 25th and 75th percentiles. The open dots represent the outlier if data was above upper whiskers or below lower whiskers. LMS: Lund–Mackay score, SNOT-22: Sino-Nasal Outcome Test-22, AQLQ: Asthma Quality of Life Questionnaire, before: before surgery, 12m after: 12 months after surgery

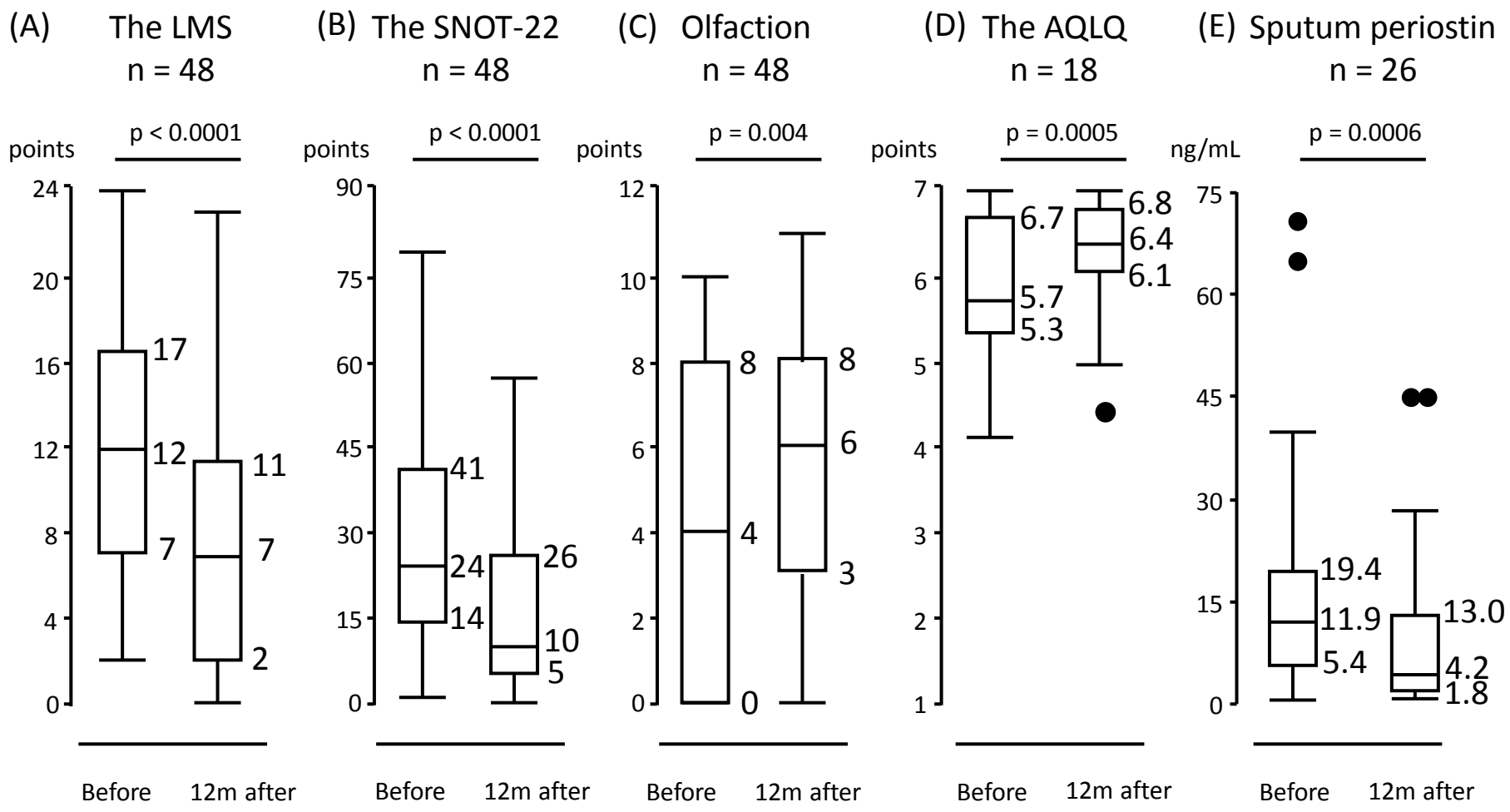
(A) The LMS
n = 48
points
p < 0.0001
24
20
16
12
8
4
0
17
12
7
11
7
2
Before
12m after
(B) The SNOT-22
n = 48
points
p < 0.0001
90
75
60
45
30
15
0
41
24
14
26
10
5
Before
12m after
(C) Olfaction
n = 48
points
p = 0.004
12
10
8
6
4
2
0
8
4
0
8
6
3
Before
12m after
(D) The AQLQ
n = 18
points
p = 0.0005
7
6
5
4
3
2
1
6.7
5.7
5.3
6.8
6.4
6.1
Before
12m after
(E) Sputum periostin
n = 26
ng/mL
p = 0.0006
75
60
45
30
15
0
19.4
11.9
5.4
13.0
4.2
1.8
Before
12m after

**Figure 1**

**Table 1. Table 1. The proportion of sensitized allergens**

| | ≥0.35 UA/mL | | | | | | ≥0.10 UA/mL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CRS patients (n = 56) | Asthma (n = 20) | W/o asthma (n = 36) | Healthy subjects (n =20) | p value* | p value** | CRS patients (n = 56) | Asthma (n = 20) | W/o asthma (n = 36) | Healthy subjects (n =20) | p value* | p value** |
| House dust mite | 19 (34) | 10 (50) | 9 (25) | - | - | 0.08 | 27 (48) | 13 (65) | 14 (39) | - | - | 0.09 |
| Dog dander | 3 (5) | 1 (5) | 2 (6) | - | - | >0.99 | 8 (14) | 4 (20) | 4 (11) | - | - | 0.44 |
| Cat | 2 (4) | 0 (0) | 2 (6) | - | - | 0.53 | 7 (13) | 0 (0) | 7 (19) | - | - | 0.04 |
| Japanese cedar | 29 (52) | 12 (60) | 17 (47) | - | - | 0.41 | 32 (57) | 13 (65) | 19 (53) | - | - | 0.41 |
| Mixed Gramineae | 7 (13) | 4 (20) | 3 (8) | - | - | 0.23 | 10 (18) | 5 (25) | 5 (14) | - | - | 0.47 |
| Mixed weed | 9 (16) | 5 (25) | 4 (11) | - | - | 0.26 | 14 (25) | 6 (30) | 8 (22) | - | - | 0.53 |
| *Alternaria* | 5 (9) | 4 (20) | 1 (3) | 1 (5) | >0.99 | 0.0497 | 12 (21) | 8 (40) | 4 (11) | 1 (5) | 0.16 | 0.02 |
| *Aspergillus* | 5 (9) | 4 (20) | 1 (3) | 1 (5) | >0.99 | 0.0497 | 8 (14) | 6 (30) | 2 (6) | 2 (10) | >0.99 | 0.02 |
| Moulds | 7 (13) | 6 (30) | 1 (3) | 2 (10) | >0.99 | 0.006 | 14 (25) | 10 (50) | 4 (11) | 2 (10) | 0.21 | 0.003 |
| SEA | 4 (7) | 1 (5) | 3 (8) | 0 (0) | 0.57 | >0.99 | 9 (16) | 5 (25) | 4 (11) | 6 (30) | 0.20 | 0.26 |
| SEB | 5 (9) | 1 (5) | 4 (11) | 0 (0) | 0.32 | 0.64 | 13 (23) | 7 (35) | 6 (17) | 3 (15) | 0.54 | 0.19 |
| SEs (A and/or B) | 6 (11) | 2 (10) | 4 (11) | 0 (0) | 0.33 | >0.99 | 15 (27) | 9 (45) | 6 (17) | 8 (40) | 0.27 | 0.03 |

*Compared between all CRS patients and healthy subjects. **Compared between asthma and w/o asthma. Eight healthy subjects could not measure serum specific IgE titers because of shortage of sample amount.

**Table 2. Participants characteristics**

| | All participants (n = 84), except where noted | Moulds+ (n = 14) | Moulds- (n = 42) | SEs+ (n = 15) | SEs- (n = 41) | Healthy subjects (n =28) | p value[*] | p value[**] |
|---|---|---|---|---|---|---|---|---|
| **General indices** | | | | | | | | |
| Age, years | 60 (50, 67) | 62 (44, 66) | 61 (51, 67) | 63 (50, 66) | 60 (51, 68) | 59 (42, 67) | 0.61 | 0.68 |
| Sex, females, n (%) | 35 (42) | 3 (21)[††] | 16 (46) | 2 (13)[††] | 17 (41) | 16 (57) | 0.06 | 0.01 |
| Body Mass Index, kg/m$^2$ | 23.3 (20.6, 25.3) | 24.0 (20.1, 26.3) | 23.5 (21.5, 25.1) | 22.7 (19.9, 26.0) | 23.9 (21.9, 25.5) | 21.6 (20.6, 24.3) | 0.59 | 0.43 |
| Smoking history, never, n (%) | 45 (54) | 7 (50)[††] | 16 (38)[†††] | 5 (33)[††] | 18 (44)[†††] | 22 (79) | 0.003 | 0.003 |
| Pack-years[‡] | 15.5 (5.3, 30) | 10 (1.6, 26.3) | 20 (7.3, 30) | 17.5 (8.1, 30.4) | 16.3 (5, 30) | 14 (4.9, 32) | 0.50 | 0.97 |
| **Diseases indices** | | | | | | | | |
| Past history of ESS, n (%)[§] | 11 (20) | 3 (21) | 8 (19) | 4(27) | 7 (17) | - | - | - |
| Presence of asthma, n (%)[§] | 20 (36) | **10 (71)[†]** | 10 (24) | **9 (60)[†]** | 11 (27) | - | - | - |
| Duration of sinusitis, years[§] | 3 (1, 10) | 2.5 (1, 8) | 3.5(1, 10) | 2 (1, 7.5) | 3.5 (1, 10) | - | - | - |
| asthma, years[¶] | 5 (1, 12) | 11 (4, 13) | 3 (1, 6) | 2.5 (1, 6) | 10 (3, 14) | - | - | - |
| ICSs dose, µg a day[¶] | 450 (340, 640) | 520 (380, 680) | 450 (240, 640) | 500 (360, 840) | 400 (320, 640) | - | - | - |
| GINA2015 Treatment step, n, (2/3, 4)[¶] | 10,10 | 4, 6 | 6, 4 | 4, 5 | 6, 5 | - | - | - |
| Conventional allergens sensitization, n (%)[§] | | | | | | - | - | - |

[*]Compared among moulds+, moulds- and H using Kruskal-Wallis test or chi-square test, [**]Compared among SEs+, SEs- and H using Kruskal-Wallis test or chi-square test, [†]**p < 0.05 for moulds or SEs + vs moulds or SEs-**, [††]p < 0.05 for moulds or SEs + vs H, [†††]p < 0.05 for moulds or SEs - vs H, [†, ††, †††]Analysed by Steel-Dwass analysis, Wilcoxon rank sum test or Fischer's exact test,[‡]n = 46(mould+/-: 7/26, SEs+/-: 10/23, H: 6), [§]n = 56,[¶]n = 20 (mould+/-: 10/10, SEs+/-: 9/11), mould: Alternaria and Aspergillus, SEs, Staphylococcus enterotoxins A and B, conventional allergens: house dust mite, dog dander, cat, Japanese cedar, mixed Gramineae, and mixed weed. H: healthy subjects

**Table 3. The impact of sensitization to moulds/SEs (≥0.10 UA/mL) on clinical outcomes**

| | All participants (n = 84) except where noted | moulds/SEs+ (n = 19) | moulds/SEs- (n = 37) | Healthy subjects (n =28) | p value* | p value** moulds/SEs + *vs* - | p value** moulds/SEs+ *vs* H | p value** moolds/SEs- *vs* H |
|---|---|---|---|---|---|---|---|---|
| **Systemic biomarkers** | | | | | | | | |
| Blood eosinophils count, /µL† | 246 (143, 526) | 449 (191, 737) | 198 (117, 361) | - | - | 0.01 | - | - |
| Serum total IgE, IU/mL†‡ | 137 (26, 431) | 513 (389, 1020) | 69 (24, 172) | 44 (11, 357) | 0.004 | <0.0001 | 0.0002 | 0.99 |
| Serum periostin, ng/mL†‡ | 86 (74, 108) | 112 (93, 159) | 80 (73, 97) | 84 (74, 101) | 0.009 | 0.008 | 0.01 | 0.95 |
| **Upper airways markers** | | | | | | | | |
| Sinus eosinophils, /HPF‡ | 66 (20, 168) | 100 (56, 178) | 60 (12, 156) | - | - | 0.17 | - | - |
| Nasal polyps, presence (%)†§ | 38 (68) | 15 (79) | 23 (62) | - | - | 0.24 | - | - |
| , eosinophils, HPF§¶ | 85 (6, 145) | 125 (65, 262) | 33 (1, 97) | - | - | 0.007 | - | - |
| Eosinophilic CRS, presence (%)† | 33 | 15 (79) | 18 (49) | - | - | 0.04 | - | - |
| Lund-Mackay scores, points† | 12 (7, 16) | 14 (9,17) | 11 (7, 16) | - | - | 0.41 | - | - |
| SNOT-22, points | 15 (3, 35) | 35 (23, 53) | 22 (12, 41) | 2 (0, 4) | <0.0001 | 0.21 | <0.0001 | <0.0001 |
| Open essence scores, points | 7 (3, 9) | 4 (0, 8) | 5 (1, 8) | 9 (7, 10) | <0.0001 | 0.98 | 0.002 | <0.0001 |
| **Lower airways markers** | | | | | | | | |
| AQLQ, points†† | 5.8 (5.5, 6.7) | 5.9 (5.2, 6.7) | 5.6 (5.5, 6.7) | - | - | 0.91 | - | - |
| Sputum eosinophils, %‡‡ | 0 (0, 3.2) | 5.5 (1.8, 57.5) | 0 (0, 2.8) | 0 (0, 0.4) | 0.0005 | 0.01 | 0.0006 | 0.29 |
| periostin, ng/mL‡‡ | 7.1 (1.5, 16.3) | 23.0 (11.7, 42.9) | 9.0 (2.0, 14.9) | 1.6 (0.5, 3.4) | <0.0001 | 0.004 | <0.0001 | 0.001 |
| FeNO, ppb | 25.8 (17.7, 38.7) | 41.7 (27.9, 73.8) | 26.2 (18.4, 38.0) | 20.6 (16.1, 26.2) | 0.0003 | 0.04 | 0.0001 | 0.13 |

*Analysed by Kruskal-Wallis test, **Analysed by Steel-Dwass analysis, Wilcoxon rank sum test or Fischer's exact test,‡n = 76 (CRS/H: 56/20), §n = 54 (moulds/SEs+/-: 18/36),¶n = 38 (moulds/SEs+/-: 15/23), ††n = 20 (moulds/SEs+/-: 13/7), ‡‡n = 65 (moulds/SEs +/-: 15/30, H: 20), moulds:

Alternaria and Aspergillus, SEs, Staphylococcus enterotoxins A and B, H: healthy subjects, Eosinophilic CRS: defined when eosinophils in sinus or NP tissue show ≥70 HPF, AQLQ: Asthma Quality of Life Questionnaire, FeNO: Fractional nitric oxide (One patient could not measure FeNO because of apparatus failure)

**Table 4. Longitudinal changes in clinical outcomes in CRS patients when stratified according to the presence or absence of sensitization to moulds/SEs (≥0.10 UA/mL)**

| | moulds/SEs+ (n = 15) | | | moulds/SEs- (n = 33) | | | |
|---|---|---|---|---|---|---|---|
| | Before | 12 months after | p value* | Before | 12 months after | p value* | p value** |
| **Systemic biomarkers** | | | | | | | |
| Blood eosinophils count, /µL | 449 (262, 653) | 341 (203, 487) | 0.08 | 178 (85, 310) | 177 (80, 317) | 0.42 | 0.10 |
| Serum periostin, ng/mL | 112 (93, 159) | 102 (64, 125) | 0.36 | 80 (72, 100) | 86 (69, 111) | 0.80 | 0.16 |
| Sensitization to moulds, n (%) | 11(73) | 10 (67) | - | 0 (0) | 0 (0) | - | - |
| Sensitization to SEs, n (%) | 11 (73) | 7 (47) | - | 0 (0) | 6 (18) | - | - |
| **Upper airways markers** | | | | | | | |
| Nasal polyps recurrence, presence, n (%)† | - | 4 (27) | - | - | 3 (9) | - | 0.18 |
| Lund-Mackay scores, points | 14 (10, 15) | 9 (6,13) | 0.004 | 11 (7, 16) | 5 (2, 11) | <0.0001 | 0.81 |
| SNOT-22, points | 31 (22, 51) | 24 (5, 32) | 0.03 | 20 (12, 33) | 10 (5, 20) | 0.0003 | 0.80 |
| Open essence scores, points | 4 (0, 8) | 6 (0, 9) | 0.06 | 5 (0, 8) | 6 (4, 8) | 0.08 | 0.67 |
| **Lower airways markers** | | | | | | | |
| New asthma onset, presence, n (%)‡ | - | 1 (25) | - | - | 6 (24) | - | >0.99 |
| AQLQ, points§ | 5.9 (4.9, 6.7) | 6.4 (6.0, 6.7) | 0.02 | 5.6 (5.5, 6.57) | 6.2 (6.1, 7) | 0.03 | 0.82 |
| Sputum eosinophils, %¶ | 3.3 (0.9, 27.2) | 3.4 (0.5, 12.1) | 0.76 | 0 (0, 2.0) | 1.0 (0, 4.5) | 0.09 | 0.33 |
| periostin, ng/mL¶ | 27.2 (14, 47.8) | 8.1 (1.7, 20.8) | 0.02 | 9.8 (2.3, 15.6) | 3.3 (1.8, 9.0) | 0.004 | 0.24 |
| FeNO, ppb†† | 50.4 (33.6, 73.8) | 32.7 (24.6, 54.6) | 0.56 | 26.4 (17.6, 38.0) | 26.3 (15.4, 46.0) | 0.86 | 0.34 |

*Analysed by Wilcoxon single rank test, **Analysed by Wilcoxon rank sum test or Fischer's exact test,†n = 34 (moulds/SEs+/-: 12/22), ‡n = 30 (moulds/SEs+/-: 5/25),§n = 18 (moulds/SEs +/-: 10/8), ¶n = 39 (moulds/SEs +/-: 13/26), ††n = 47 (moulds/SEs +/-: 14/33), moulds: Alternaria and Aspergillus, SEs, Staphylococcus enterotoxins A and B, AQLQ: Asthma Quality of Life Questionnaire, FeNO: Fractional nitric oxide

**Supplemental Materials**

# Moulds and *Staphylococcus aureus* enterotoxins are relevant allergens to affect type2-inflammation in CRS patients

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**Table E1. The influence of interaction between moulds and SEs sensitization for clinical outcomes in CRS patients**

| | F value | p value |
|---|---|---|
| The prevalence of asthma | 6.39 | 0.01 |
| **Systemic biomarkers** | | |
| Blood eosinophils count, /μL | 0.20 | 0.66 |
| Serum total IgE, IU/mL | 1.71 | 0.20 |
| Serum periostin, ng/mL | 4.24 | 0.04 |
| **Upper airways biomarkers** | | |
| Sinus eosinophils, /HPF | 0.06 | 0.81 |
| Nasal polyp, presence | 7.50 | 0.006 |
| Nasal polyps, eosinophils, HPF[†] | 0.65 | 0.43 |
| Eosinophilic CRS, presence | 4.02 | 0.045 |
| Lund-Mackay scores, points | 2.27 | 0.14 |
| SNOT-22, points | 0.47 | 0.49 |
| Open essence scores, points | 0.98 | 0.32 |
| **Lower airways biomarkers** | | |
| AQLQ, points[*] | 0.81 | 0.38 |
| Sputum eosinophils, %[†] | 4.95 | 0.03 |
| periostin, ng/mL[†] | 0.60 | 0.44 |
| FeNO, ppb | 0.11 | 0.74 |

Eosinophilic CRS: defined when eosinophils in sinus or NP tissue show ≥70 HPF, [*]n = 20, [†]n = 45, AQLQ: Asthma Quality of Life Questionnaire, FeNO: Fractional nitric oxide (One patient without moulds/SEs sensitization could not measure FeNO because of apparatus failure

**Table E2. The impact of sensitization to moulds/SEs (≥0.35 UA/mL) on clinical outcomes**

| | All participants (n = 84) | moulds/SEs+ (n = 11) | moulds/SEs- (n = 45) | Healthy subjects (n =28) | p value* | p value** Alt/SEs+ *vs* Alt/SEs- | p value** Alt/SEs+ *vs* H | p value** Alt/SEs- *vs* H |
|---|---|---|---|---|---|---|---|---|
| **Systemic biomarkers** | | | | | | | | |
| Blood eosinophils count, /µL† | 246 (143, 526) | 419 (167, 653) | 228 (135, 478) | - | - | 0.22 | - | - |
| Serum total IgE, IU/mL† | 137 (26, 431) | 569 (389, 1580) | 116 (27, 212) | 44 (11, 357) | 0.0003 | 0.0005 | 0.002 | 0.67 |
| Serum periostin, ng/mL† | 86 (74, 108) | 93 (78, 138) | 84 (73, 116) | 84 (74, 101) | 0.45 | 0.75 | 0.42 | 0.73 |
| **Upper airways markers** | | | | | | | | |
| Sinus eosinophils, /HPF‡ | 66 (20, 168) | 82 (16, 335) | 64 (20, 156) | - | - | 0.57 | - | - |
| Nasal polyps, presence (%)† | 38 (68) | 8 (73) | 30 (67) | - | - | >0.99 | - | - |
| , eosinophils, HPF§ | 85 (6, 145) | 135 (77, 291) | 77 (4, 126) | - | - | 0.03 | - | - |
| Eosinophilic CRS, presence (%)† | 33 (59) | 7 (64) | 26 (58) | - | - | >0.99 | - | - |
| Lund-Mackay scores, points† | 12 (7, 16) | 14 (7,16) | 11 (7, 17) | - | - | 0.80 | - | - |
| SNOT-22, points | 15 (3, 35) | 46 (29, 68) | 23 (12, 36) | 2 (0, 4) | <0.0001 | 0.052 | <0.0001 | <0.0001 |
| Open essence scores, points | 7 (3, 9) | 4 (0, 9) | 5 (0, 8) | 9 (7, 10) | <0.0001 | 0.96 | 0.04 | <0.0001 |
| **Lower airways markers** | | | | | | | | |
| AQLQ, points¶ | 5.8 (5.5, 6.7) | 6.2 (4.5, 6.8) | 5.6 (5.5, 6.3) | - | - | 0.55 | - | - |
| Sputum eosinophils, %†† | 0 (0, 3.2) | 2.3 (0.5, 35.2) | 0.3 (0, 7.0) | 0 (0, 0.4) | 0.02 | 0.67 | 0.02 | 0.06 |
| periostin, ng/mL†† | 7.1 (1.5, 16.3) | 19.3 (9.8, 39.0) | 11.7 (3.7, 21.0) | 1.6 (0.5, 3.4) | <0.0001 | 0.24 | 0.0002 | 0.0001 |
| FeNO, ppb‡‡ | 25.8 (17.7, 38.7) | 39.4 (24.7, 57.4) | 29.0 (21.6, 50.6) | 20.6 (16.1, 26.2) | 0.004 | 0.64 | 0.01 | 0.01 |

*Analysed by Kruskal-Wallis test, **Analysed by Steel-Dwass analysis, Wilcoxon rank sum test or Fischer's exact test, †n = 56, ‡n = 76 (CRS/H: 56/20) n= 54 (moulds/SEs+/-: 10/44), §n = 38(moulds/SEs+/-: 8/30), ¶n = 20 (moulds/SEs +/-: 7/13), ††n = 65 (moulds/SEs +/-: 8/37, H: 20),‡‡n = 83, §§n = 45. moulds: Alternaria and Aspergillus, SEs, Staphylococcus enterotoxins A and B, H: healthy subjects, Eosinophilic CRS: defined when

eosinophils in sinus or NP tissue show ≥70 HPF, AQLQ: Asthma Quality of Life Questionnaire, FeNO: Fractional nitric oxide (One patient without moulds/SEs sensitization could not measure FeNO because of apparatus failure)