Inflammatory patterns in upper airway disease in the same geographical area may change over time

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ABSTRACT

Background: *Inflammatory patterns of nasal polyps (NPs) may vary. Changes over time have not been investigated so far. This study was designed to evaluate the inflammatory patterns of NPs in Thailand at two time points 12 years apart, explore differences in* Staphylococcus aureus *(SA) mucosal carriage rates over time, and the latter's relationship with the inflammatory patterns.*

Methods: *Formalin-fixed nasal tissue was obtained from 89 (47 in 1999 and 42 in 2011) patients suffering from chronic rhinosinusitis with NPs (CRSwNPs). Tissues were evaluated for eosinophils, neutrophils, IgE$^+$ cells, IgE and macrophage mannose receptors, interleukin (IL)-5 and IL-17 cytokine profile, and the presence of SA, using automated immunohistochemistry and peptide nucleic acid–fluorescence* in situ *hybridization.*

Results: *We found a significant increase in the absolute values of eosinophils and IgE$^+$ cells in the 2011 CRSwNP tissue series compared with 1999 and a significant but smaller increase in neutrophils. Semiquantitative evaluation revealed significantly higher mean values of positive cells for all studied inflammatory markers in the 2011 group of patients, except for the high-affinity IgE receptor. This "eosinophilic shift" of inflammation was accompanied by higher SA carriage, as well as higher frequencies of SA invasion (54.8% versus 10.6%; $p < 0.001$) in the 2011 compared with 1999 subjects. Patients with asthma were more likely to have higher SA carriage rates compared with nonasthmatic patients.*

Conclusion: *There was a shift from predominantly neutrophilic to eosinophilic CRSwNPs in Thai patients within 12 years, with an increase in various inflammatory markers including IgE, which is associated with an increase in intramucosal presence of SA.*

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Chronic rhinosinusitis with nasal polyps (CRSwNPs) has been proven to represent a chronic severe inflammatory disease of the upper airways, often associated with comorbid asthma, with distinct inflammatory and remodeling profiles.[1–3] In white subjects, the mucosal inflammatory process mainly is orchestrated by Th2 cytokines and is characterized by an increased tissue eosinophilia and a local IgE production, which may be amplified by *Staphylococcus aureus* enterotoxins (SEs).[1–7] *Staphylococcus aureus* (SA), through the superantigen activities of SEs, has been found to be an important disease modifier with SE–IgE formed in about one-half of NPs in Europe, a factor associated with asthma comorbidity.[7–15] An increase in alternatively activated macrophage mannose receptor–expressing macrophages, indicating a disturbed phagocytosis and intracellular killing of SA; in IgE expressing cells; as well as an increase in cells expressing the low (CD23)- and high (FceRI)-affinity IgE receptors has been reported in these polyp samples.[16] In Asian patients, however, the predominant T-effector cell in polyps has been identified as the Th17 cell, with interleukin (IL)-17 as the key cytokine resulting in a predominance of neutrophils.[7,9,10] Only a minor part of polyp samples from central China were IL-5$^+$ and expressed SE–IgE.[7] We also have observed differences in the inflammatory patterns between the coastal region and central China[9,10] and a difference in the colonization of polyps with either predominantly Gram-positive or Gram-negative bacteria in IL-5$^+$ versus IL-5$^-$ samples.[17]

With these differences, we hypothesized that the inflammatory patterns of CRSwNPs may not only vary with the location, but also over time. The fact that about two-thirds of polyp patients recently investigated in Tokyo/Japan were IL-5$^+$ and ~52% expressed SE–IgE, although polyps were described to be mainly neutrophilic in the past, further supported this assumption.[18] However, no data are available to confirm changes in the inflammation pattern in CRSwNP Asian patients over time.

The aim of this study, consequently, was (i) to evaluate the current inflammatory patterns of NPs in a developing Asian city, Bangkok, and compare those to the patterns in polyp samples collected 12 years ago; (ii) to explore differences in SA carriage rates and intramucosal SA presence over time and to study their relationship with the inflammatory patterns; and (iii) to explore differences in the characteristics of inflammation and intramucosal SA in asthmatic Thai patients versus nonasthmatic Thai patients.

MATERIALS AND METHODS

NP tissue samples from 89 Thai patients were taken out of the archives of the Department of Otorhinolaryngology of the Mahidol University Hospital in Bangok, Thailand, after charts were reviewed that fulfilled the inclusion criteria of the study; all patients underwent primary surgical intervention for CRSwNPs from a single surgeon, during the first 6 months of the years 1999 or and 2011. Forty-seven patients (26 males, 21 females, mean age: 33.55 ± 15.05 years) were identified in the year 1999 ("1999" group) and 42 patients (24 males and 18 female patients; mean age, 47.33 ± 13.67 years) were identified

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Table 1 **Number of eosinophils and IgE(+) cells (median [IQR]) and number of patients (%) with SA grading, as well as size of edema, in the two groups of patients and in asthmatic patients vs nonasthmatic patients**

	Patients		*p* Value	Asthma		*p* Value
	Group 1999	Group 2011		No	Yes	
Eosinophils (nr; median [IQR])	5.00 (5–15)	35.00 (5–95)	0.002*	15.00 (5–50)	35.00 (7.5–65)	0.137*
IgE (nr; median [IQR])	3.90 (2.4–5.5)	5.60 (2.6–10.9)	0.018*	4.50 (2.2–8.2)	5.70 (3.5–8.1)	0.227*
SA (nr of patients [%])			<0.001#			<0.001#
Negative	33 (70.2)	11 (26.2)		40 (51.9)	4 (33.3)	
Noninvasive	9 (19.2)	8 (19.0)		17 (22.1)	0 (0.0)	
Invasive	5 (10.6)	23 (54.8)	<0.001#	20 (26.0)	8 (66.7)	0.005#
Positive	14 (29.8)	31 (73.8)	<0.001#	37 (48.1)	8 (66.7)	0.230#
Edema (nr of patients [%])			0.033#			0.085#
No	5 (10.6)	1 (2.4)		4 (5.2)	2 (16.7)	
Mild	24 (51.1)	14 (33.3)		36 (46.8)	2 (16.7)	
Severe	18 (38.3)	27 (64.3)		37 (48.1)	8 (66.7)	

**Mann-Whitney test.*
#Chi-square test.
SA = Staphylococcus aureus; *IQR = interquartile range; nr = number.*

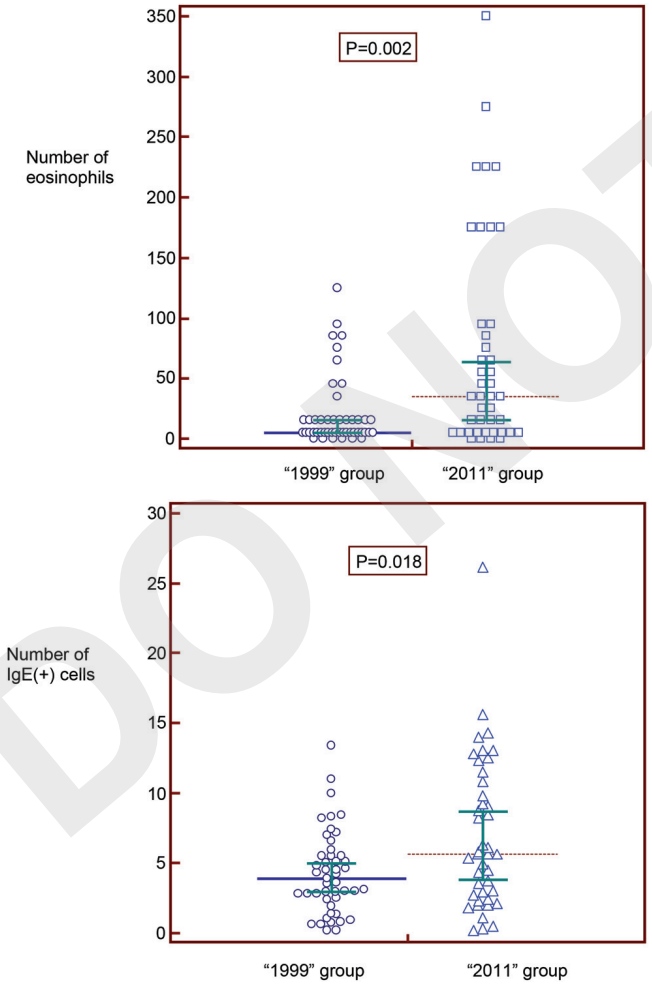

Figure 1. *Number of eosinophils and IgE(+) cells in the 1999 and 2011 group of patients. Data are expressed as medians and 95% confidence intervals (CIs).*

in 2011 ("2011" group). The approach of using two different Thai populations of primary NPs was necessary to exclude the impact of surgery on the results.

The diagnosis was based on history, clinical examination, nasal endoscopy, sinus computed tomography scanning, skin-prick testing (SPT) for atopy, and test of pulmonary function. All patients fulfilled the criteria of bilateral NPs according to the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines.[19] Computed tomography scans were graded according to Lund-Mackay.[20] Polyps were graded by size and extent in both the left and the right nasal cavity on a scale of 0–3, according to the Davos classification.[19] The atopic status was evaluated by SPTs to common inhalant allergens. The diagnosis of asthma was performed by a chest physician according to Global Initiative for Asthma 2006 guidelines based on symptoms and pulmonary function tests.[21] Patients were asked not to use nasal and oral steroids, antihistamines, antileukotriene, and antibiotics 4 weeks before surgery, according to the protocol we applied for the treatment of CRSwNP patients independently of the study. All patients gave their written informed consent and the study protocol was approved by the Institutional Ethics Committee of Mahidol University Hospital.

Automated Immunohistochemistry

Automated immunohistochemistry (IHC) was performed using the BOND-MAX system (Leica Microsystems GmbH, Wetzlar, Germany). Five-micron sections were prepared from formalin-fixed, paraffin-embedded nasal tissues and mounted on SuperFrost Plus glass slides (Menzel Gläser, Braunschweig, Germany). These slides were covered by Bond Universal Covertiles (Leica Microsystems). All procedures were performed automatically in seven steps according to the manufacturer's instructions.[22]

In this protocol the following primary antibodies were used for the evaluation of eosinophils, IgE^+ cells, CD23, FceRI and macrophage mannose receptors, IL-5 and IL-17 cytokines, and myeloperoxidase (MPO) for neutrophils: IgE (A0094, 1/1500 dilution; Dako, Glostrup, Denmark), IL-5 (MAB605, 1/1000 dilution; R&D Systems Abingdon, United Kingdom), CD23 (NCL-C-CD23–1B12, 1/20 dilution; Leica), MannoseR (ab117644, 1/500 dilution; Abcam, Cambridge, United Kingdom), FceRI (ab54411, 1/400 dilution; Abcam), and MPO (A0398, 1/3000 dilution; Dako). For the staining of IL-17, we incubated in step 3 with a polyclonal goat primary antibody (AF-317-NA, 1/50; R&D Systems) and in step 4 we incubated with a biotinylated polyclonal rabbit anti-goat immunoglobulin secondary antibody (E0466, 1/500 dilution; DakoCytomation, Glostrup, Denmark).

Samples were analyzed in 10 serial sections by using a magnification of 400× and were scored by two independent observers unaware of the diagnosis and clinical data. Edema and eosinophil counts were evaluated on hematoxylin and eosin–stained sections. Edema was

semiquantitatively scored on a three-point scale (0 represented the lowest and 2 the highest scores). For eosinophils and IgE cells we counted the absolute values of positive cells per field.

Similarly, for the number of positive cells for MPO, IL-5 and IL-17 cytokines, CD23, FceRI, and mannose receptors, a scale from 0 to 3 was applied (0, 1–10, 11–20, and >20 cells/field, respectively).

Identification of SA by Peptide Nucleic Acid-Fluorescence In Situ Hybridization

Nasal mucosa tissue paraffin sections (5-μm) were deparaffinized. After air-drying, the sections were hybridized at 55°C for 90 minutes in a humidified chamber with 100–500 nM of fluorescein-labeled PNA probe (SA Peptide Nucleic Acid-Fluorescence In Situ Hybridization [PNA-FISH] kit; AdvanDx, Woburn, MA) as described previously.[8,23]

The staining was scored from 0 to 3: 0, negative; 1, no invasion, superficial presence; 2, intraepithelial presence; and 3, subepithelial, intracellular presence. Ten high-power fields were counted and added resulting in a total score between 0 and 30. Furthermore, we defined SA invasion as negative (all 10 fields scored 0), noninvasive (at least 1 field scored 1), or invasive (at least 1 field scored 2 or 3) based on the maximum score. Two independent observers read the slides.

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), Version 19.0 (SPSS, Inc., Chicago, IL). Age was expressed as the mean ± SD. Normally distributed inflammatory markers were expressed as the means and standard error (SE), and nonnormally quantitative inflammatory markers were expressed as medians and interquartile ranges; they were analyzed using *t*-test and Mann-Whitney, respectively. All of the other variables were categorical; they were expressed as frequencies and percentages (%) and were analyzed using the chi-square test. The normality of quantitative variables was ascertained with the Kolmogorov-Smirnov test. The Spearman's *r* bivariate correlation was used to assess the statistical correlation among parameters. Multivariate linear and nominal logistic regression analysis was used to explore the independency of effects of time/group on the studied parameters when adjusting for age. All tests were two tailed and statistical significance was considered for values of $p < 0.05$.

RESULTS

Although selected based on primary surgery for CRSwNPs within a specified time frame, patients in the 2011 group were significantly older than patients in the 1999 group ($p < 0.001$), and the two groups were comparable in terms of gender ($p = 0.863$). The number of patients positive for at least one of the most common aeroallergens was similar in both groups (16 [38.1%] in 2011 versus 16 [34.0%] in 1999; $p = 0.691$). More patients in the 2011 group presented with an asthma history than in the 1999 group (8 [19.0%] versus 4 [8.5%]; $p = 0.146$), although not statistically significant. There were no patients in the 1999 group with aspirin intolerance based on history, whereas there were three patients in the 2011 group ($p = 0.062$). Finally, there was no difference in the severity of disease between 1999 and 2011 groups, with >80% of samples f ograde 2 and 3 of polyps in both years.

IHC Results

The absolute numbers of eosinophils and IgE (+) cells were significantly higher in the 2011 versus the 1999 CRSwNP tissue series (35

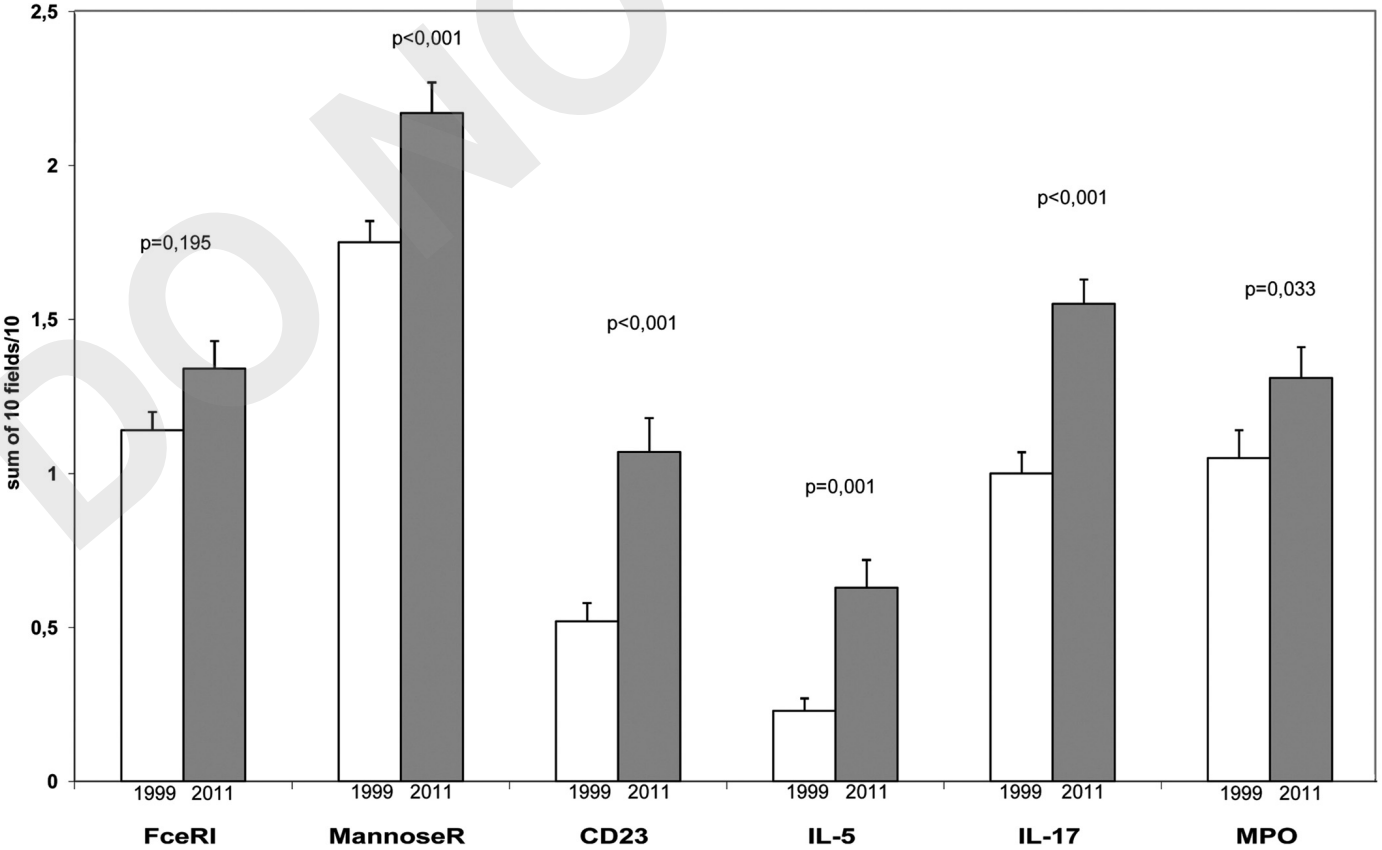

Figure 2. Semiquantitative evaluation of positive cells for FceRI, Mannose, CD23 receptors, interleukin (IL)-5, IL-17 cytokines, and myeloperoxidase (MPO). The sum of 10 fields/10 was calculated for each subject and values are reported as means + standard error (SE). Shown are the 1999 (white bars) and 2011 group of patients (gray bars).

versus 5, $p = 0.002$, and 5.6 versus 3.9, $p = 0.018$, respectively; Table 1; Fig. 1). Semiquantitative evaluation revealed that significantly higher numbers of positive cells were present in the 2011 group of patients for mannose receptor (2.17 ± 0.10 versus 1.75 ± 0.07; $p < 0.001$), CD23 (1.07 ± 0.11 versus 0.52 ± 0.06; $p < 0.001$), IL-5 (0.63 ± 0.09 versus 0.23 ± 0.04; $p = 0.001$), and IL-17 cytokine (1.55 ± 0.08 versus 1.00 ± 0.07; $p < 0.001$) compared with the 1999 subjects (Fig. 2). A small increase was also observed for neutrophils over time in the two groups of patients (1.31 ± 0.10 versus 1.05 ± 0.09; $p = 0.033$; Fig. 2). No difference was found for FceRI receptor expression (1.34 ± 0.09 versus 1.14 ± 0.06; $p = 0.195$; Fig. 2).

A significantly higher number of samples from the 2011 group were positive for SA bacteria compared with the samples from the 1999 series (73.8% versus 29.8%; $p < 0.001$; odds ratios [ORs] = 6.64; 95% confidence interval [CI] = 2.62–16.83; Table 1; Fig. 3), also reflected by higher total scores (median [interquartile range]) in the 2011 group compared with the 1999 group ($0\frac{0}{2}$ versus 9 [0–19.5]; $p < 0.001$; Fig. 4). Invasion of the mucosa by SA was significantly more frequent in the 2011 group of patients compared with 1999 subjects (54.8% versus 10.6%; $p < 0.001$; OR = 10.17; 95% CI = 3.36–30.81; Table 1). Representative slides of eosinophils, IgE^+ cells, CD23, and mannose receptor, IL-5 cytokine expressing cells, and PNA-FISH for SA in the 1999 and 2011 CRSwNP patient series are presented in Fig. 5.

Within each group, we covered a wide span of years (13–68 years for 1999 and 13–75 years for 2011 group). Because there was a significant difference of mean age between the two groups, we investigated whether age was an important predictor for IHC parameters. However, there was no relationship for any parameters with age (eosinophils, Spearman's $r = 0.066$, $p = 0.541$; IgE(+), $r = 0.017$, $p = 0.875$; MPO, $r = 0.025$, $p = 0.813$; FceRI, $r = 0.069$, $p = 0.521$; CD23, $r = 0.219$, $p = 0.039$; MannoseR, $r = 0.053$, $p = 0.620$; IL-5, $r = 0.222$, $p = 0.036$; IL-17, $r = 0.036$, $p = 0.740$; and SA, $r = 0.271$, $p = 0.010$). We also performed multivariate linear and nominal logistic regression analyses to control for the possible confounding effect of age. There was no impact of the patient's age on any findings, and significant differences between groups persisted after adjustment for subjects' age.

Furthermore, no association was found between the number of eosinophils and IgE(+) cells and gender or SPT results. Similarly, no statistically significant association was found for MPO, FceRI, MannoseR, CD23, IL-5, or IL-17 with gender and SPT results. Finally, no association was found between presence of SA and gender or SPT results.

Edema formation was more severe in the 2011 versus the 1999 CRSwNPs series ($p = 0.033$; Table 1). No or mild edema was observed in the vast majority of 1999 samples, whereas severe edema was found in the majority of the 2011 group.

Finally, asthmatic patients were more likely to have higher SA colonization rates ($p < 0.001$) compared with nonasthmatic patients (Table 1). The number of eosinophils and IgE^+ cells in patients with asthma was not significantly different from nonasthmatic patients ($p = 0.137$ and $p = 0.227$ respectively; Table 1). It is worth mentioning, however, that all asthmatic patients were positive for eosinophils and IgE cells. With respect to SA invasion, invasion was observed significantly more frequently in asthmatic patients compared with nonas-

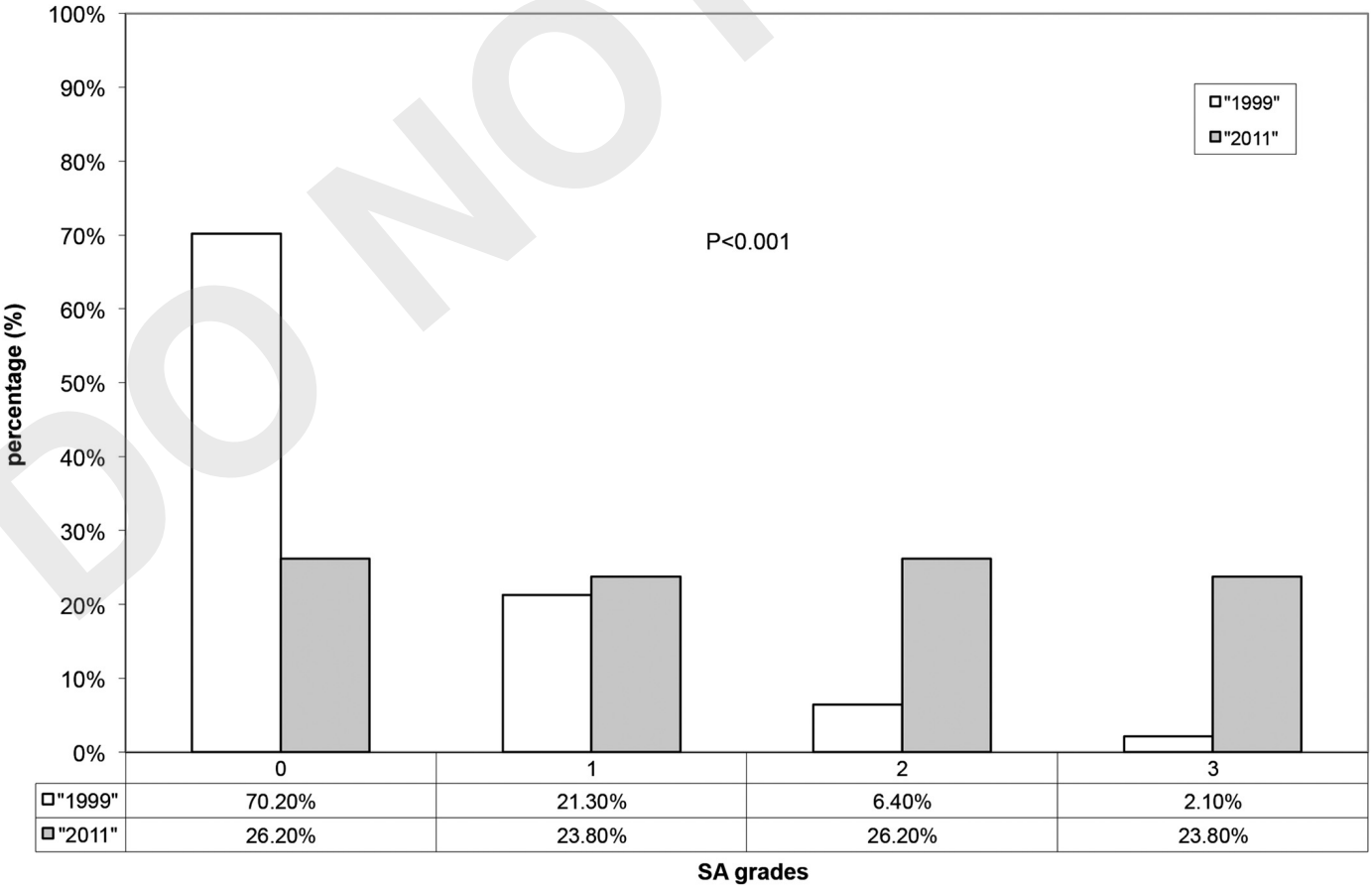

Figure 3. *Percentage of patients per mucosal invasion grade of* Staphylococcus aureus *(SA) in 1999 and 2011 groups. A semiquantitative scoring system was used to quantify the relative amount of SA present in the tissue. The intensity of SA staining, by means of peptide nucleic acid–fluorescence* in situ *hybridization (PNA-FISH) technique was scored from 0 to 3 (0, negative; 1, no invasion; superficial presence; 2, intraepithelial presence; and 3, subepithellial, intracellular presence).*

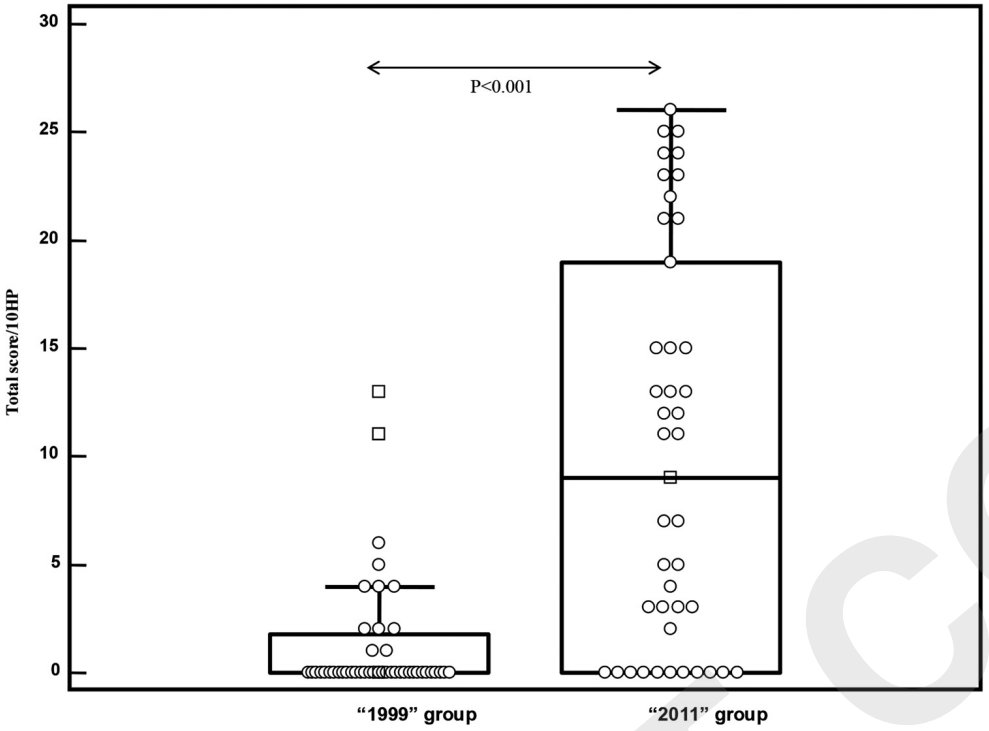

Figure 4. Total scores for presence of Staphylococcus aureus *(SA) in 1999 and 2011 groups, detected by peptide nucleic acid–fluorescence* in situ *hybridization (PNA-FISH). To take into account the number of SA bacteria in the tissue, 10 high-power fields were counted and scores were added resulting in a total score between 0 and 30. Data for each patient in the two distinct times/groups (1999 and 2011) are expressed as median (interquartile range [IQR]).*

thmatic patients (66.7% versus 26.0%; $p = 0.005$; OR = 5.70; 95% CI = 1.55–20.99; Table 1). When considering only nonasthmatic patients, the significant differences described previously between the two groups, 1999 and 2011, remained.

DISCUSSION

This is the first study to provide evidence that changes in inflammatory patterns of airway disease may occur over time and that this inflammatory shift is associated with a change in bacterial presence, here, of SA. The results of this study support recent observations of Asian colleagues that polyps have been more neutrophilic in the past decades but seem to have changed in inflammatory character over the last years.[9–11] We already have observed differences between continents and within China between the coastal developed region and more remote mainland and hypothesized that there may be a change not only with the location, but also over time.[9,10] Thus, we evaluated inflammatory markers including granulocyte predominance, IgE and IgE receptor expression, cytokine expression, and a marker for alternative activation of macrophages in NP tissues collected at two different time periods in Bangkok, Thailand.

The approach of using two different Thai populations of primary NPs collected within defined time windows 12 years apart was the only possible approach. First, it would not be ethical or feasible to follow patients for 10 years or more without providing surgery, if indicated; second, we wanted to exclude a negative selection of disease (only the very mild patients would stay without surgery over that time) or the impact of surgery, if performed during this time, on the results (any change in cell numbers could be attributed to surgery rather than time). Although the patients were selected within fixed time frames based on their first surgery for NPs, there was an unexpected difference in the average age between groups. A possible explanation of this difference might be the referral system in Thailand, which has been changed in-between the two collection periods. Although 10 years ago patients could directly visit the specialist, patients today need to visit their primary care doctor before coming to a tertiary care center, possibly delaying surgery. However, although this could have resulted in more severe cases coming to the hospital in 2011, in our study populations there was no difference in the severity of disease. Furthermore, we have to point out that by using appropriate statistical methods, we have excluded the impact of age on inflammatory patterns or presence of mucosal SA and shown that our observations are true changes over time.

This study confirms the predominantly neutrophilic character of CRSwNPs 12 years ago and the change into a predominantly eosinophilic inflammatory pattern today, associated with an increase in the mucosal presence of SA. We found significantly increased numbers of eosinophils and IgE(+) cells in the 2011 NP tissues compared with those of 1999, with a significant correlation between them. There was a sevenfold increase in the median absolute values for eosinophils in the two groups of patients, whereas in the same time the increase in mean values for neutrophils was small (1.2-fold), further shifting the ration eosinophils/neutrophils over 1.

This increase also was paralleled by a striking increase in remodeling in terms of edema formation. It is interesting to mention that no association was found for the number of eosinophils and IgE(+) cells with age and SPT results; thus, local expression of IgE^+ cells was not related to inhalant allergy or to aging of the patients.[4,24] This confirms former findings of dissociation between SPTs and locally formed IgE in airway mucosal disease.[4] The shift to an eosinophilic type of inflammation in Thai polyp patients goes together with significantly higher numbers of IL-5 cytokine-positive cells in the 2011 compared with the 1999 CRSwNP group. Furthermore, in line with the increase in IgE^+ cells, the low-affinity IgE receptor showed significantly higher expression. CD23 is a B-cell–specific antigen that plays an essential role in B-cell growth and differentiation and the up-regulation of IgE production.[25] Thus, the up-regulation of CD23 in the 2011 group would be consistent with elevated total IgE concentrations.[1,4]

Mucosal macrophages are known to be located at the interface with the external environment. Depending on the factors the macrophages encounter, macrophages become polarized into a classically activated proinflammatory (M1) phenotype or into an alternatively activated (M2) phenotype.[16] Alternatively activated macrophages are immunosuppressive, propagate Th2 polarized immune responses and contribute to the recruitment of polarized Th2 cells and eosinophils; they also

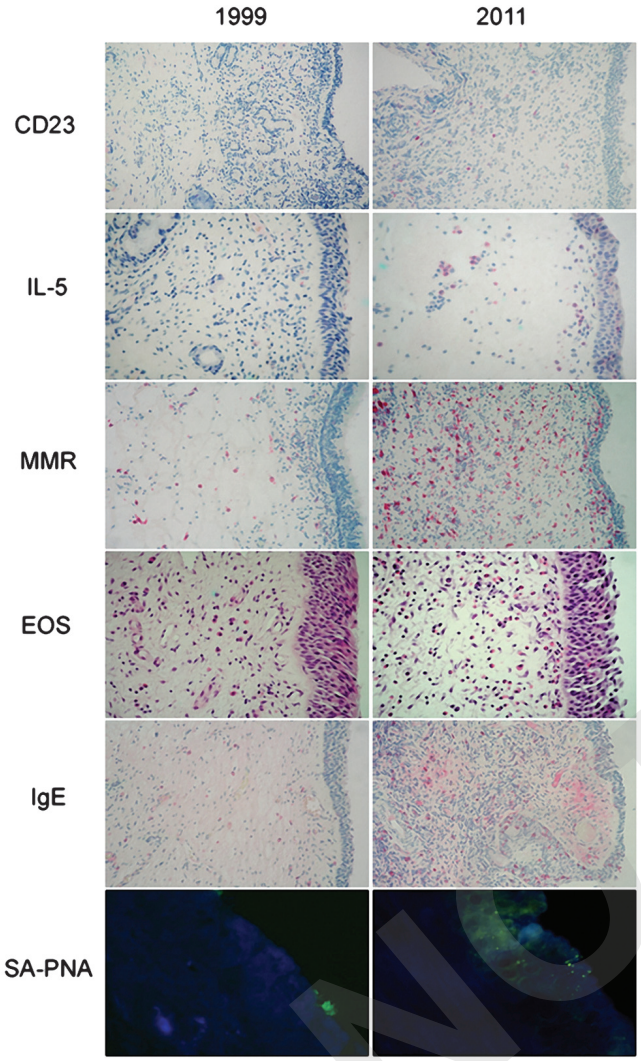

Figure 5. Automated immunohistochemistry (IHC)-stained and peptide nucleic acid–fluorescence in situ hybridization (PNA-FISH) sections of nasal polyp tissue (NP) samples. Representative slides of eosinophils, IgE^+ cells, CD23, and mannose receptor, IL-5 cytokine-expressing cells and PNA-FISH for Staphylococcus aureus *(SA) in the 1999 and 2011 chronic rhinosinusitis with NP (CRSwNP) patient series. Pictures are taken at a magnification of 200 or 400×. PNA-FISH images were using a fluorescein isothiocyanate (FITC) filter (63× magnification).*

support intracellular survival of bacteria and viruses. In our study we confirmed former findings and found elevated numbers of alternatively activated macrophages in line with a shift toward a Th2-biased inflammatory pattern in the recent CRSwNP group.[16,26,27] Th2 cytokines such as IL-5 may alternatively activate macrophages, and we previously showed that IL-5 would specifically reduce the capacity of killing intracellular germs.[16] Thus, in an IL-5–expressing environment such as a predominantly eosinophilic NP, macrophages might be ineffective in defending the mucosa against invading bacteria.

To our knowledge, here, we describe for the first time changes in mucosal SA carriage over time and explore these changes in relation to the changes observed at the inflammatory level. Using PNA-FISH, we were able to detect intramucosal SA, in line with recent insights that CRSwNPs is characterized by high rates of SA colonization, and staphylococcal products such as enterotoxins may amplify the mucosal inflammation.[1–15] In this study, we show that a significantly higher number of 2011 patients show presence of intramucosal SA compared with their 1999 counterparts. It is not clear, however, to what extent SA only profits from the deficit in mucosal defense to increase its mucosal presence[16] and/or actively drives the associated changes in the inflammatory patterns in the direction of a Th2 bias, as described earlier.[28] An "environmentally-based" factor for this "eosinophilic shift" in inflammatory patterns in Thai patients, also resulting in a significant increase in mucosal SA carriage over time, may be a possible explanation of the results. Moreover, such a factor would be in accordance with studies that support the significant impact of environmental factors (*e.g.*, air pollution, infectious agents and host factors, and smoking habits) on CRSwNP pathogenesis.[2,29] Additional studies are needed to clarify this relationship.

We have already described that IgE antibodies to staphylococcal superantigens within the polyp tissue considerably increase the risk of suffering from comorbid asthma.[7] Here, we found that asthmatic patients were more likely to show mucosal SA colonization as well as intracellular presence compared with nonasthmatic patients. Although we were technically not able to detect SE–IgE in the fixed histological sections, these findings are in agreement with the concept of intramucosal presence of SA, releasing enterotoxins and amplifying the inflammatory response to an extent that is associated with comorbid asthma.[7,14,15]

CONCLUSION

Here, we show for the first time a unique potential shift from neutrophilic to eosinophilic inflammation patterns in CRSwNP Thai patients, associated with an increase in intramucosal IgE and SA. These findings support former observations on the close link between Th2 biased inflammation and SA and its enterotoxins with comorbid asthma. The findings also underline that the dogma "NPs are eosinophilic" probably needs to be revised not only with respect to the differences formerly observed in white versus Asian patients, but also with respect to changes over time within a population. This may be attributed to an "environmentally based" factor and could be evidence for a hygiene hypothesis variant. This has relevance for the prognosis and the treatment of patients with CRSwNPs.

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