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Age-associated changes in chronic rhinosinusitis endotypes

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Abstract

Background: Immunological function in innate and adaptive immunity changes with the aging process. Thus, age-related cytokine profiles in chronic rhinosinusitis (CRS) need to be investigated for precision medicine.

Objective: The objective of this study was to characterize age-related changes in immunologic profiles according to CRS subtypes.

Methods: Subjects in control (n = 29), CRS without nasal polyps (CRSsNP, n = 86), and CRS with nasal polyps (eosinophilic NP: ENP, n= 81; non-eosinophilic NP: NENP, n=113) were enrolled in this study. Twenty markers for type 1/2/3 inflammation and other inflammatory processes were measured in homogenates of sinonasal tissues and statistically analyzed.

Results: In control tissues, type 2/3 and proinflammatory mediators showed an inverse correlation with age. CRSsNP and NENP showed an age-related increase in type 2 cytokines and a decline in type 3 cytokines. Interestingly, the age-related decrease in type 3 mediators was associated with those of CT scores in NENP. ENP showed an age-related increase in type 3 cytokines with type 2 mediators sustained at high levels. Smokers with ENP demonstrated age-associated increases in type 1/2/3 mediators as well as CT scores. These age-related patterns in each CRS were confirmed by statistically adjusting atopy status, smoking history, and disease duration.

Conclusion: Age-associated cytokine changes differed among CRS subtypes and control tissues. CRSsNP and NENP demonstrated a decline in type 3 mediators and increase in type 2 mediators, whereas type 3 mediators increased with age in ENP.

Keywords: Age, Cytokine, Endotype, Rhinosinusitis

INTRODUCTION

Chronic rhinosinusitis (CRS) is a sinonasal inflammatory disease associated with asthma comorbidity that affects around 10% of the population worldwide.[1] CRS is diagnosed based on clinical symptoms and objective findings such as endoscopy and computed tomography (CT).[2] Despite simple diagnostic criteria, CRS is a very heterogeneous disease that is attributable to its multifactorial etiologies.[2, 3] Among these multiple etiologies, age may be one of factors influencing CRS pathophysiology, considering that age is a strong predictor of human immunity to external stimuli including viral infections.[4] Immunological function in innate and adaptive immunity changes with the aging process.[5] Distinct age-related distributions of immune cells were observed in peripheral blood: plasma cells reach maximum counts in early life, followed by memory B cells and immunoglobulin heavy chain isotypes.[6] Moreover, a gradual deterioration of immune function with age termed immunosenescence,[7] might affect the pathophysiology of airway inflammatory diseases including CRS.[8, 9]

Our previous studies reported that CRS exhibited an age-related decline in innate barrier function in eosinophilic nasal polyps (ENP),[10] and an age-related decrement in neutrophilic inflammation in non-eosinophilic nasal polyps (NENP), which reflected treatment outcomes in elderly NENP subjects.[11] Interestingly, elderly CRS patients were characterized by increased neutrophilic proinflammatory cytokines such as IL-1 β , IL-6, IL-8, and TNF- α and

by less responsiveness to steroid treatment in a recent study.[12] These earlier findings suggest that age-related distributions of inflammatory mediators differ among CRS endotypes.

Taking into account that current pharmacologic strategies are increasingly focused on specific inflammatory pathways,[13] an understanding of the age-associated immune regulation in the nasal mucosa will be critical to establishing rational approaches to precision medicine for CRS. Therefore, we investigated age-related changes in inflammatory mediators using the sinonasal tissues of control subjects and those with CRS subtypes.

MATERIALS AND METHODS

Study design and population

Subjects classified into control (n = 29), CRS without nasal polyps (CRSsNP, n = 86), and CRS with nasal polyps (CRSwNP, n = 194) were enrolled in this study, and sinonasal tissues were collected between January 2014 and September 2016. Biospecimens for this study were provided by the Samsung Medical Center Biobank. All CRS subjects were diagnosed based on personal history, physical examination, nasal endoscopy, and CT findings of the sinuses according to the 2012 European position paper on rhinosinusitis and nasal polyp guidelines.[14] Exclusion criteria were as follows: (1) prior treatment with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs including biologics for 4 weeks before surgery, (2) history of prior sinus surgery, and (3) unilateral rhinosinusitis, antrochoanal polyps, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease. Control tissues were obtained during other rhinologic surgeries, such as skull base, lacrimal duct, or orbital decompression surgery, from patients without any sinonasal diseases. In this study, we obtained uncinate process tissues from control and CRSsNP patients and nasal polyp tissues from CRSwNP patients. CRSwNP cases were divided into eosinophilic nasal polyps (ENP) and non-eosinophilic nasal polyps (NENP), where tissue eosinophilia was

defined as an eosinophilic count exceeding 10% of the total inflammatory cells per high power field in NP tissues. Eosinophilic CRSsNP were excluded for analysis because they accounted for a small portion of CRSsNP (9.5%) and difficult to analyze in this study cohort. The atopic status of study patients was evaluated using the ImmunoCAP[®] assay (Phadia AB, Uppsala, Sweden), which detects specific IgE antibodies against common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, tree mixture, weed mixture, grass mixture, cat, and dog). Patients were considered atopic if the allergen-specific IgE level was greater than 0.35 KU/L. A diagnosis of asthma was performed by an allergist based on medical history and lung function analysis, including methacholine challenge tests. Also, asthmatic patients were excluded who used oral corticosteroid or biologics within 4 weeks prior to surgery. Disease extent was measured using Lund-Mackay CT scores[15] and endoscopic Lund-Kennedy scores,[16] and the severity of patient-reported symptoms was evaluated with the 22-item SinoNasal Outcome Test (SNOT-22).[17] Subjects were divided into four subgroups by age (< 20 years old, 20 to 39 years old, 40 to 59 years old, and $\geq$ 60 years old) to test for nonlinear associations between cytokine expression levels and age.

Tissue sampling and cytokine assays

All tissues were immediately frozen and stored at -80°C and we used samples stored less than 3 years. To analyze the cytokine profile, each tissue sample was submerged in 1 mL phosphate-buffered saline supplemented with 0.05% Tween-20 (Sigma-Aldrich, St Louis, MO) and 1% PIC (Sigma-Aldrich) per 0.1 g of tissue. This tissue was homogenized with a mechanical homogenizer, a Bullet Blender Blue (Next Advance, Averill Park, NY). After homogenization, the suspensions were centrifuged at 3,000 rpm for 10 min at 4°C. The supernatants were then separated and stored at -80°C for further analysis of cytokines and other inflammatory mediators.[18] Twenty inflammatory markers were investigated in the

nasal tissues using a multiplex cytokine assay kit (Thermo Scientific Inc., Rochester, NY). The protein concentrations of the tissue extracts were determined using the Pierce 660 nm Protein Assay Kit (Thermo Scientific Inc.). Samples were thawed at room temperature and vortexed to ensure that they were well mixed. Multiplex cytokine analysis kits (CCL-11, CCL-24, IL-5, periostin, CXCL-1, CXCL-2, CXCL-8, IL-17A, IL-22, IL-23, IL-6, IFN- γ , S100A8, IL-1 α , IL-1 β , IL-10, IL-33, IL-25, BAFF, TGF- β 1) were obtained from R&D Systems (Cat. No. LMSAHM) and data were collected using a Luminex 100 (Luminex, Austin, TX). Data analysis was performed using the MasterPlex QT version 2.0 (MiraiBio, Alameda, CA). All assays were run in duplicate according to the manufacturers' protocol. The sensitivity for each cytokine are as follows: CCL-11 (14.6 pg/mL), CCL-24 (1.34 pg/mL), IL-5 (0.5 pg/mL), periostin (95.7 pg/mL), CXCL-1 (5.3 pg/mL), CXCL-2 (7.86 pg/mL), CXCL-8 (1.8 pg/mL), IL-17A (1.8 pg/mL), IL-22 (11.7 pg/mL), IL-23 (11.4 pg/mL), IL-6 (1.7 pg/mL), IFN- γ (0.4 pg/mL), S100A8 (15.3 pg/mL), IL-1 α (0.9 pg/mL), IL-1 β (0.8 pg/mL), IL-10 (1.6 pg/mL), IL-33 (1.8 pg/mL), IL-25 (27.7 pg/mL), BAFF (1.01 pg/mL), and TGF- β 1 (2.1-24.6 pg/mL). Values of less than the detection limit were given equally to half of the lower limit.[19] All of the protein levels in the tissue homogenates were normalized to the concentration of total protein (mg/mL).[20] The Institutional Review Board of Samsung Medical Center approved this study (IRB No. SMC 2017-02-129), and informed consent was obtained.

Statistical analysis

Statistical analyses were performed using Stata software v14.0 (StataCorp LP, College Station, TX) and R version 3.5.1 (Vienna, Austria; <http://www.R-project.org/>). The Shapiro-Wilk test was used to test the normality of the distributions of all analyzed variables, and data that were not normally distributed were presented as medians and interquartile ranges. Data

were analyzed by the Mann-Whitney U test or Kruskal-Wallis test with Dunn's multiple comparison test. Fisher's exact test or the Chi-square test was used for categorical variables. Correlations were tested by Spearman's rank correlation coefficients to assess the association with aging. Cytokine data were transformed into Z-score with a mean of 0 and a standard deviation of 1, to account for the variability of each cytokine. All analyzed cytokine data for each group were displayed using locally weighted scatterplot smoothing (LOWESS) regression curve.[21] The purpose of LOWESS method is to remove noise of the raw data and to refine the graph. Z-scores of cytokine data were subdivided into different intervals for individual regression analysis. LOWESS analysis and curve were performed using the *lowess* of STATA software. In order to choose a proper analytical model that explored the relationship between age and cytokines, we focused on the volatility in the cytokine levels with aging. Also, the influences of atopy, smoking status, and symptom duration on the expression of inflammatory cytokines were considered confounding factors. The nonlinear association between cytokine expressions and age was explained by a generalized additive model (GAM) in which possible confounding variables such as atopy status, smoking history, and disease duration were linearly adjusted.[22] GAM estimated by the outcome variable in GAMs is represented as sums of the linear and nonlinear functions of the covariates:[23]

$$g(x) = s_0 + \sum_{i=1}^p n_i(x_i) + \sum_{j=1}^q l_j(x_j)$$

where s_0 : intercept; $n_1, \dots, n_p$: nonlinear function of $x_1, \dots, x_p$ and $l_1, \dots, l_q$: linear function of $x_{p+1}, \dots, x_{p+q}$. After adjusting the covariates by model, the age-related relationship with a given cytokine was visualized as marginal plots with a fitted line and 95% confidence interval area. In the GAMs, smooth lines were represented by penalized regression splines, which estimated by penalized likelihood maximization with smoothers selected by generalized cross validation. The GAM was implemented by the *mgcv* package in R. All plots

were displayed for each subgroup (Control, CRSsNP, NENP, and ENP). *P* values less than 0.05 were considered statistically significant.

RESULTS

Study population and demographics

In total, 280 CRS and 29 control samples were selected for analyzing the age-related changes in inflammatory mediators. Their clinical characteristics are shown in online supplementary Table S1 and the distribution of CRS subtypes is illustrated in Fig. S1. There were no differences in sex, asthmatic comorbidity, disease duration, and SNOT-22 among groups, whereas, blood eosinophil count, proportion of atopic status, and smoking history were higher in the ENP groups compared to the CRSsNP and NENP groups using the Dunn's multiple comparison test. The prevalence of asthma was relatively higher in ENP (9.9%) compared with CRSsNP (3.5%) and NENP (3.5%), but the difference was not statistically significant ($p = 0.242$). Patients with a history of prior sinus surgery were excluded because the immunologic profile varies between revision and primary surgery cases.[24] For this reason, the prevalence of asthma in CRS patients might be lower than previous data. The Lund-Kennedy scores were significantly different in the increasing order of the CRSsNP, ENP, and NENP groups. The Lund-Mackay CT score was the lowest in the CRSsNP group among CRS groups. There was difference in demographics such as age and smoking history between the ENP and NENP/CRSsNP groups, since CRSsNP and NENP showed bimodal distributions with peaks at the second and sixth decade, whereas the prevalence of ENP was peaked at the sixth decade, with few subjects aged less than 20 years old (Fig. S1). ENP was infrequent in the subjects under 20 years of age of our study population. These findings may be consistent with previous studies showing that childhood CRS cases were less eosinophilic.[25, 26] When CRS subgroups were further divided by age (Table 1), there were

no differences in sex, asthma comorbidity, disease duration and blood eosinophil count among age groups. The group aged less than 20 years had a lower proportion of smoking history or atopic status, and higher endoscopic or CT scores in CRSsNP and NENP, compared with the other age groups.

Correlation between age and inflammatory mediators in each CRS subtype

Twenty inflammatory mediators of each CRS subtype were evaluated using multiplex cytokine assays: type 2 (IL-5, CCL-11, CCL-24, and periostin), type 3 (IL-17A, CXCL-1, CXCL-2, CXCL-8, IL-22, IL-23, and IL-6), type 1 (INF- γ), proinflammatory (S100A8, IL-1 α , IL-1 β , and IL-10), epithelial-derived innate (IL-33 and IL-25) and other inflammatory (TGF- β 1 and BAFF) mediators. To investigate the age-related changes in the cytokines of each CRS group, we performed correlation analyses (Fig. 1). In control tissues, diverse lineages of cytokines such as type 2 mediators (CCL-11 and periostin), type 3 mediators (IL-17A, CXCL-8, and IL-6), epithelial-derived innate cytokines (IL-33 and IL-25), S100A8, and TGF- β 1, showed an inverse correlation with aging (Fig. S2A). These downregulated cytokines were correlated with each other (Fig. S2B). However, BAFF was upregulated with aging and showed a trend of an inverse relationship with TGF- β 1 ($r = -0.3913$, $p = 0.0586$). Declines in an immune tolerance marker and increases in a B-cell inflammatory marker in elderly control tissues might reflect immunosenescence suggesting susceptibility to autoimmunity.

However, CRS tissues showed much different age-related cytokine patterns, compared with control tissues. Overall, all of the CRS mucosa ($n = 280$), type 2 mediators including IL-5, CCL-11, CCL-24, and periostin increased with aging, whereas type 3 mediators showed a slight decrease with aging in CRS tissues (Fig. 1A, second column). NENP showed age-related increases in type 2 cytokines such as IL-5 and CCL-24 and age-related declines in

type 3-associated cytokines such as CXCL-1 and CXCL-8. Interestingly, aging was inversely associated with CT scores in NENP (Fig. 1A, fourth column). In NENP, CT scores were positively correlated with type 3-associated mediators such as IL-17A ($r = 0.2446$, $p = 0.0090$), CXCL-1 ($r = 0.3326$, $p = 0.0006$), CXCL-8 ($r = 0.2582$, $p = 0.0058$), IL-22 ($r = 0.2497$, $p = 0.0082$), IL-1 β ($r = 0.2929$, $p = 0.0030$), but were negatively correlated with type 2 mediators such as periostin ($r = -0.3842$, $p = 0.0001$) and IL-25 ($r = -0.2235$, $p = 0.0173$). These findings signify that the age-related decline in type 3-associated cytokines affects the disease extent in NENP where type 3 mediators play the main role.[20]

CRSsNP demonstrated a similar pattern of the B-cell inflammatory markers from control tissues, and their patterns of type 2 and type 3 mediators were similar to in NENP (Fig. 1A, third column). ENP exhibited age-related increases in type 3-associated cytokines such as IL-17A and CXCL-1 (Fig. 1A, fifth column). Lastly, IFN- γ increased with aging in all types of CRS. Notably, there were differences in the age-related changes of inflammatory mediators between ENP and the other subtypes of CRS such as NENP and CRSsNP.

Levels of cytokines which showed significant age-related changes in each CRS, were transformed into Z-score with a mean of 0 and a standard deviation of 1 to better understand the relationship among type 1, 2 and 3 cytokines in each CRS subtype (Fig. 1B). In CRSsNP and NENP, type 1 (green color) and type 3 (blue series color) were predominant from childhood to around 60-year-old but there was equivalence or reversal between type 1/3 and type 2 (red series color) at elderly age. In ENP, type 2 mediators were maintained at higher levels for all the ages and type 3 mediators showed a subtle increase through life-time. IFN- γ reached plateau at middle or old age, then dropped after 60-year-old.

Correlation between age and inflammatory mediators in each CRS subtype according to the presence of atopy or history of smoking

Because the presence of atopy and smoking history may affect CRS endotype,[27-29] stratified analyses were performed on the age-related changes of each CRS subtype. In the ENP groups, the age-related changes in inflammatory mediators consistently demonstrated that type 3 (IL-17A) and type 1 (IFN- γ) increased with aging, irrespective of atopy status or smoking history (Fig. 2A). Moreover, smokers with ENP showed age-related increases in type 2 cytokines (IL-5, CCL-11, and CCL-24) as well as type 1 and type 3 mediators, and also demonstrated age-related increases in disease extent based on CT scan ($r = 0.3427$, $p = 0.0548$). Of note, type 3 and proinflammatory mediators may play more important roles in ENP with smoking history, given that CT scores seemed to be associated with IL-17A ($r = 0.3288$, $p = 0.0662$), CXCL-8 ($r = 0.5763$, $p = 0.0006$), IL-1 α ($r = 0.3631$, $p = 0.0575$) and IL-1 β ($r = 0.4143$, $p = 0.0284$), but not with IL-5 ($r = 0.0225$, $p = 0.9208$).

In CRSsNP and NENP groups, non-smoker and non-atopy groups showed an age-related increase in type 2 mediators and decline in type 3, which is similar to the unstratified data (Fig. 2B and 2C). However, the atopy status or smoking history in CRSsNP and NENP offset the age-related changes in inflammatory mediators. These findings suggest that in CRSsNP and NENP, various clinical factors may contribute to age-associated immunologic changes.

Age-related nonlinear pattern in inflammatory mediators in CRS adjusted by the presence of atopy, history of smoking, and disease duration

Cytokine levels vary with age, and those of age-related changes revealed irregular pattern. Therefore, nonlinear effects of aging were statistically evaluated with adjustments for atopy status, smoking history, and disease duration using GAM (Fig. 3-5). GAM is an analytic method that can identify whether the increase or decrease of cytokine level is statistically

significant in a certain range of age, in addition to the gradually increasing or decreasing pattern. Because disease duration might be associated with age, it was included in adjustment lists for CRS subtype.[30, 31]

In control tissues, type 3 (IL-17A, CXCL-8, and IL-6) or proinflammatory (S100A8), type 2 (CCL-11), and regulatory cytokine (TGF- β 1) showed maximum levels during childhood and decreased progressively until young adult age (20-30 years), remaining relatively stable thereafter (Fig. 3A). The level of IL-33 decreased progressively with aging in control tissues (Fig. 3B).

In the CRSsNP group, type 3 mediators (CXCL-8 and IL-6) exhibited maximum levels during childhood as in control tissues, and declined progressively until adulthood (20 years), maintaining stable levels from that age on (Fig. 4A). TGF- β 1 gradually decreased with age, whereas periostin and BAFF increased continuously with age (Fig. 4B). Strikingly, there was a distinct cytokine pattern at 20-39 years old. Inflammatory markers such as IL-25 and IL-1 α peaked at 33 and 34 years old, respectively, whereas anti-inflammatory cytokine, IL-10, showed a maximum level at 34 years of age (Fig. 4C). There were no differences in sex, atopic status, asthmatic comorbidity, smoking history, blood eosinophil percent, or symptom scores between the 20- to 39-year-old subgroup and the other age subgroups of CRSsNP. However, lesser endoscopic scores were observed in the 20- to 39-year-old age group (Table 1). These findings might indicate that an anti-inflammatory effect, such as IL-10 overpowered pathologic ones at this range of age (20-39 years old).

In the NENP group, CCL-24 demonstrated a gradual and continued increase consistently throughout the subjects' lifetime. IFN- γ levels fluctuated until subjects were 40 years old, reached the maximum level in the sixth decade, and decreased progressively after that. The level of IL-33 was peaked at 60 years, and those of BAFF and CXCL-1 tended to decrease with age (Fig. 5A).

In the ENP group, IL-17A increased gradually and continuously with aging, CXCL-1 showed a gradual increase from the age of 50-60 years, and CCL-24 surged as subjects neared age 70 years (Fig. 5B). Additionally, major cytokines such as IL-5, CCL-11, and periostin showed no age-related changes in ENP.

According to this study, age-related cytokine characteristics consisted of four patterns: a pattern under 20 years of age, a pattern over 60 years of age, a continuously changing pattern, and a specific age-related pattern (Fig. S3). These age-related cytokine patterns are summarized in Table 2.

DISCUSSION

There have been few studies demonstrating age-related cytokine changes in healthy sinonasal tissues and CRS subtypes. Cho *et al.* reported that the level of eosinophil cationic protein in nasal lavage fluid was negatively correlated with age in CRSwNP patients and the level of S100A8 and S100A9, affecting epithelial barrier function, was decreased according to aging in CRS patients and normal subjects.[9, 10] Our group previously showed an age-related decline of neutrophil infiltration and expression of IL-17A and IL-23 in NENP.[11] The limitations of these studies were the analysis of a relatively small study population and no adjustment of confounders. To address a gap in knowledge, this study analyzed age-related changes, as well as statistically adjusting for major confounding factors such as atopy, smoking, and disease duration, since they are strongly associated with CRS and NP.[29] This study may be the first to present age-related cytokine changes in local inflammatory tissues of each CRS subtype and to suggest clinical implications with a relatively large population.

Type 1- and type 3-associated mediators and epithelial cytokines were downregulated with increasing age in control tissues. In detail, our prominent findings are that subjects aged less than 20 years old showed upregulation of multiple lineages of cytokines compared with

other age groups: type 2 (CCL-11 and periostin), type 3 (IL-17A, CXCL-8, and IL-6), proinflammatory (S100A8), epithelial-derived (IL-33 and IL-25), and regulatory cytokine (TGF- β 1) (Fig. S2). When adjustments were made for confounding factors such as atopy and smoking, IL-17A, CXCL-8, IL-6, CCL-11, S100A8, and TGF- β 1 were uniquely increased under 20-year-old group. Children and adolescence subjects are susceptible to recurrent viral upper airway infections compared with adults.[32] Type 3-associated cytokines (IL-17A, CXCL-8, and IL-6) and regulatory cytokine (TGF- β 1) play a role in the innate defense system.[33] IL-17-producing T cells are abundant in healthy mucosal tissues as well as NP tissues as compared with peripheral blood T cells.[34] A local type 3 inflammatory response may be important in healthy nasal mucosal immune homeostasis.[34] CCL-11 was upregulated only in control tissues of the under 20-year-old group and seemed to play a role as a counteractor of CXCL-8 since it was reported that CCL-11 suppresses production of the neutrophil chemoattractant CXCL-8 and plays a role as a negative regulator of neutrophil recruitment.[35] Interestingly, CRSsNP subjects aged under 20 years old demonstrated higher expression levels of CXCL-8 and IL-6, like the control group, but CCL-11 was not upregulated. These findings suggest that a balance between CCL-11 and CXCL-8 may prevent overzealous inflammatory responses and maintain biological homeostasis

IL-33 is expressed constitutively at steady-state and functions as an alarm in response to infection or allergy.[36] Both cytokines play a role in the defense mechanisms of healthy mucosa but may be senescent, since age-related decline in immune function has been observed in components of the innate immune response, including epithelial cell-derived defense molecules and neutrophils.[8]

When adjustments were made for atopy status, smoking history, and disease duration, the CRSsNP group, which was characterized by type 1 or type 3 inflammatory responses,[19] showed intriguing findings. A type 3 mediators (CXCL-8 and IL-6) showed higher levels at

under 20-year-old than the other older ages (Fig. 4A). Interestingly, in CRSsNP subjects aged under 20-year-old, the Lund-Kennedy endoscopic scores were higher than in older age groups (Table 1). Changes in type 3 mediators may be related to disease severity in CRSsNP. This hypothesis might also be applied to the other type 3-associated CRS, NENP.[37] Age-related decline in type 3-associated cytokines (CXCL-1 and CXCL-8) and their association with CT scores suggest similar clinical implications. However, when adjusted for atopy status, smoking history, and disease duration, a decline in type 3 mediators was not apparent in NENP except for CXCL-1. These findings suggest that multiple factors are involved in age-related cytokine changes in nasal polypogenesis. Of note, both CRSsNP and NENP showed consistent increases in type 2 cytokines (IL-5, CCL-24, or periostin). However, the clinical significance of age-related increase of type 2 cytokines in these CRSs remains unclear.

The ENP group showed uniquely characteristic features compared with the other subtypes of CRS and control tissues. When adjusted for atopy status, smoking history, and disease duration, type 3 mediators such as IL-17A increased continuously with aging and CXCL-1 surged after age 60. This finding is supported by a recent study showing that mucus profiles in elderly CRS patients (older 60 years old) were characterized by exaggerated neutrophilic inflammatory responses, which were associated with clinical infection.[12] In addition to upper airway inflammation, the underlying inflammation of elderly asthmatics are different from those of younger patients and are less type 2-associated.[38] The other feature of age-related cytokine changes in the ENP group is that smoking history significantly affected the age-related endotype in ENP, whereas atopy status didn't (Fig. 2A). Type 2 mediators (IL-5, CCL-11, and CCL-24) increased with age in the ENP subjects with a smoking history. Moreover, TGF- β 1, a marker for regulatory T cells, was downregulated with aging. As a consequence of these changes, disease extent increased with aging in smokers of the ENP group. Smoking is well-known to be an independent predictor for poor long-term outcomes

after endoscopic sinus surgeries.[39] In our previous study utilizing eosinophilic CRS murine models, smoking exposure enhanced eosinophilic infiltration and generated more severe polypoid remodeling with type 1, 2, and 3 responses upregulated.[28] The CRS mucosal cytokine profile of smokers has been reported to be dominated type 3 mediators such as IL-17A, CXCL-8, and TNF- α in earlier studies.[40, 41] Dysregulated host defenses such as reduced expression level of the polymeric immunoglobulin receptor, secretory leukocyte protease inhibitor, and the long and short palate, lung and nasal epithelium clone protein were observed when cigarette smoke was exposed.[42] Given that barrier function declines and type 3 inflammatory process increases particularly in elderly eosinophilic CRS,[10, 12] this age-related CRS pathophysiologic mechanism could be aggravated by smoking exposure.

The shortcoming of this cross-sectional study causes a selection bias that participants were recruited from a tertiary referral hospital. A prospective study using a longitudinal cohort is warranted to confirm the effects of age on immunological changes in the sinonasal mucosa. Also, treatment outcomes should be considered according to the age groups. Previous articles regarding the impact of age on surgical outcomes using SNOT-22 reported that elderly patients with CRS had lower symptom burden [43, 44] but showed different results: lesser improvement [43] versus similar result in elderly after endoscopic sinus surgery.[44] These studies suggested that age should be incorporated into CRS treatment algorithms. Unfortunately, they did not perform CRS endotyping which may influence immunologic reactions as implicated from our study. Age-associated immunologic changes may be related to clinical symptom burden and treatment outcomes. Therefore, further well-designed research is needed to confirm previous findings. Another shortcoming of this study is that the statistical significance is not strong enough, particularly correlation coefficients of heatmaps. In the NENP group, most of correlation coefficients were around 0.3 because NENP includes very heterogeneous subtypes such as neutrophilic, lymphocytic, and plasma

cell-dominant subtypes.[43] Thus, we applied an additional statistical method, GAM, to reveal the non-linear effect of age on inflammatory mediators.

In summary, age-related changes in CRS endotypes varied according to CRS subtypes. CRSsNP and NENP groups showed an age-related decline in type 3 mediators reflecting disease severity, in contrast, ENP demonstrated an age-related increase in type 3 mediators with type 2 mediators sustained at high levels. Clinical characteristics such as atopy status and smoking history may affect age-related endotype changes. Smoking may be one of the factors influencing the age-related increase in inflammatory mediators in ENP. In some cases, age-related changes in CRS disease severity can be associated with those in inflammatory markers.

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Figure Legends

Figure 1. A heat map of correlation analysis between age and cytokines. Colored cells are statistically significant ($p < 0.05$) using Spearman's rank correlation coefficient. The color bar presents a correlation coefficient (Spearman's rho) that red color represents positive correlation and green color represents negative correlation. (A) A heat map of all subjects according to phenotypes. (B) Locally weighted scatterplot smoothing curves of Z-score of major cytokines according to ages in control and CRS subtypes. Major cytokines (IL-5, IL-17A, and IFN- γ) and additional markers (CCL-11 or CCL-24 for type 2 inflammation and CXCL-1 or CXCL-8 for type 3 inflammation) are selected. Among the additional markers,

cytokines with statistically significant or high correlation coefficient in each subgroup are presented.

Figure 2. A heat map of ENP, CRSsNP, and NENP subjects according to the presence of atopy or history of smoking. Colored cells are statistically significant ($p < 0.05$) using Spearman's rank correlation coefficient. Underlined values indicate near significant p values to being statistically significant ($0.5 < P \text{ value} < 0.6$).

Figure 3. Marginal plots of the generalized additive model with a fitted line and 95% confidence interval area (gray color). Red colored P value represents negative. (A) Age-related changes in the levels of cytokines in control tissues. Type 3 or proinflammatory mediators and regulatory cytokine showed maximum levels at ages under 20 years. (B) The level of IL-33 decreased gradually with aging in control tissues.

Figure 4. Marginal plots of CRSsNP tissues. Red colored P value represents negative correlation and blue colored P value represents negative correlation. (A) The levels of CXCL-8 and IL-6 were the highest during childhood ages, the same as in control tissues. (B) The level of TGF- β 1 gradually decreased with aging, whereas that of periostin and BAFF increased with aging. (C) Diverse lineages of cytokines including IL-1 α , IL-25, and IL-10 were upregulated at 20 to 39 years of age.

Figure 5. Marginal plots of nasal polyp tissues. Red colored P value represents negative correlation and blue colored P value represents negative correlation. (A) In NENP tissues, the level of CCL-24 gradually increased with aging, and that of IFN- γ fluctuated until 40 years

old and reached the highest level in the sixth decade. (B) In ENP tissues, IL-17A increased gradually, CXCL-1 increased after 60 years old, and CCL-24 soared after 70 years.

Figure S1. Numbers of control subjects and chronic rhinosinusitis patients according to age and subtypes. CRSsNP and NENP revealed bimodal distributions with peaks in the second and sixth decade, and the prevalence of ENP peaked at the sixth decade.

Figure S2. Correlation between age and cytokines in control subjects. (A) All lineages of cytokines were inversely correlated with aging except BAFF. (B) The heat map shows positive correlations among downregulated cytokines. Underlines indicate near significant p values ($0.5 < P \text{ value} < 0.6$).

Figure S3. Age-related relationship with cytokines is classified into four patterns.

Table 1. Demographics of chronic rhinosinusitis subtypes according to age subgroups

	CRSsNP (N=86)				NENP (N=113)				ENP (N =81)		
	< 20	20-39	40-59	> 60	< 20	20-39	40-59	> 60	20-39	40-59	> 60
No.	30	13	29	14	35	20	39	19	16	54	11
Age (y)	16	29	50	63	15	30	49	67	34.5	51	64
Male sex, %	76.7	100	75.9	57.1	60.0	90.0	82.1	63.2	75.0	72.2	100
Atopic, %	33.3	61.5	55.2	42.9	11.4* [†]	45.0*	38.5 [†]	36.8	62.5	55.6	36.4
Asthmatic, %	0	0	10.3	0	0	5.0	7.7	0	18.8	7.4	9.1
Smoking, %	6.7* [†]	23.1	48.3*	35.7 [†]	0* ^{†‡}	35.0*	41.0 [†]	31.6 [‡]	43.8	44.4	9.1
Lund-Kennedy score	6* ^{†‡}	1.5* [§]	4 ^{†§}	4 [‡]	8*	6* [‡]	6	9 [‡]	6	6	7
Lund-Mackay score	8	6	7	9.5	16*	13	11*	14	11.5	11	14
Blood eosinophil (%)	2.6	2.25	2.8	3.4	2.2	3.8	3.1	2	6.5	4.8	4.8
Disease duration (month)	65.5	31.1	46.3	72.7	41.7	68.6	122.2	47.9	53.5	60.5	99.2
SNOT-22	24	25.5	21	29	24	35	37	38	42*	30*	41

Data are expressed as medians. The same footnote indicates statistical significance on the Dunn's multiple comparison test between two groups.

CRSsNP, chronic rhinosinusitis without nasal polyp; *ENP*, eosinophilic nasal polyp; *NENP*, non-eosinophilic nasal polyp; *SNOT-22*, the 22-item SinoNasal Outcome Test

Table 2. Summary of age-related inflammatory profiles in chronic rhinosinusitis subtypes

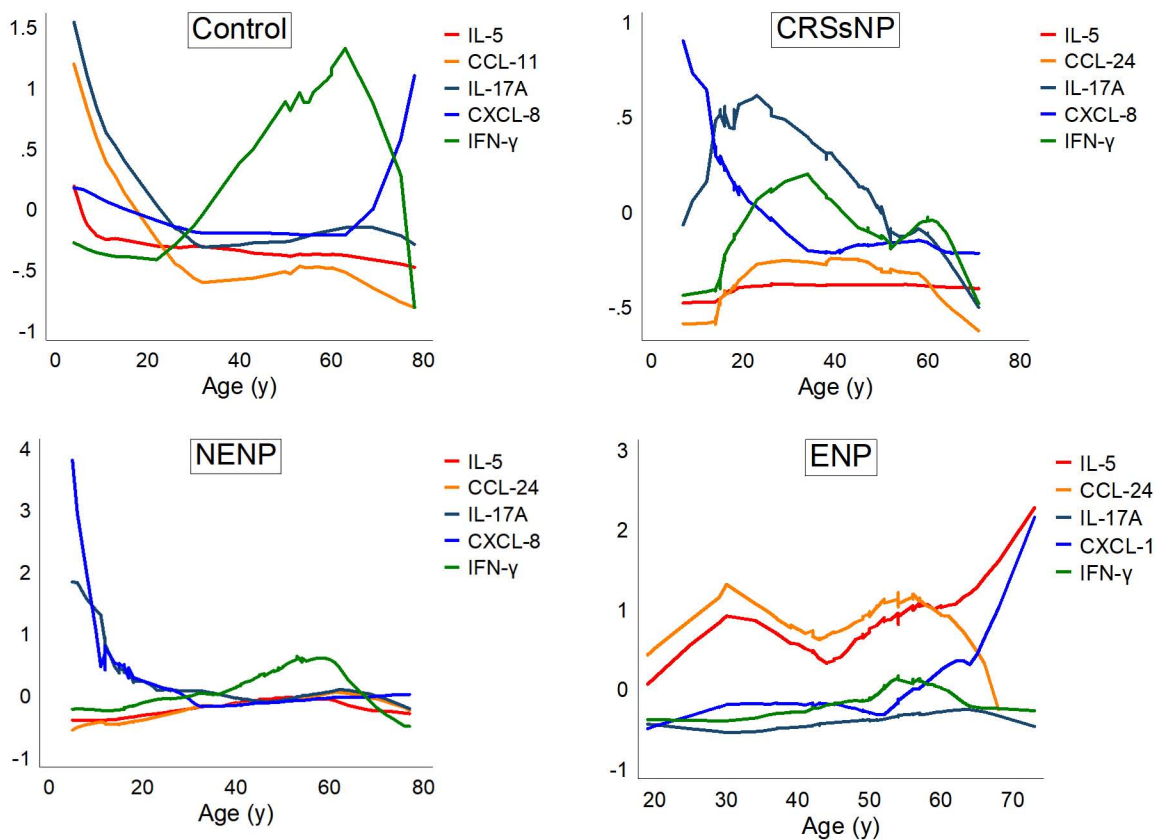
Pattern	Control	CRSsNP	NENP	ENP
Pattern under 20 years of age	CCL-11 ↓ IL-17A ↓ CXCL-8 ↓ IL-6 ↓ TGF-β1 ↓	CXCL-8 ↓ IL-6 ↓		
Pattern over 60 years of age				CCL-24 ↑ CXCL-1 ↑
Continuously changing pattern (increasing or decreasing)	IL-33 ↓	Periostin ↑ BAFF ↑ TGF-β1 ↓	CCL-24 ↑ CXCL-1 ↓	IL-17A ↑
Specific age-related pattern (range of age, y)		IL-1α ↑ IL-10 ↑ IL-25 ↑ (20-40 y)	IFN-γ ↑ (50-60 y) BAFF ↓ (40-50 y) IL-33↑ (50-65 y)	

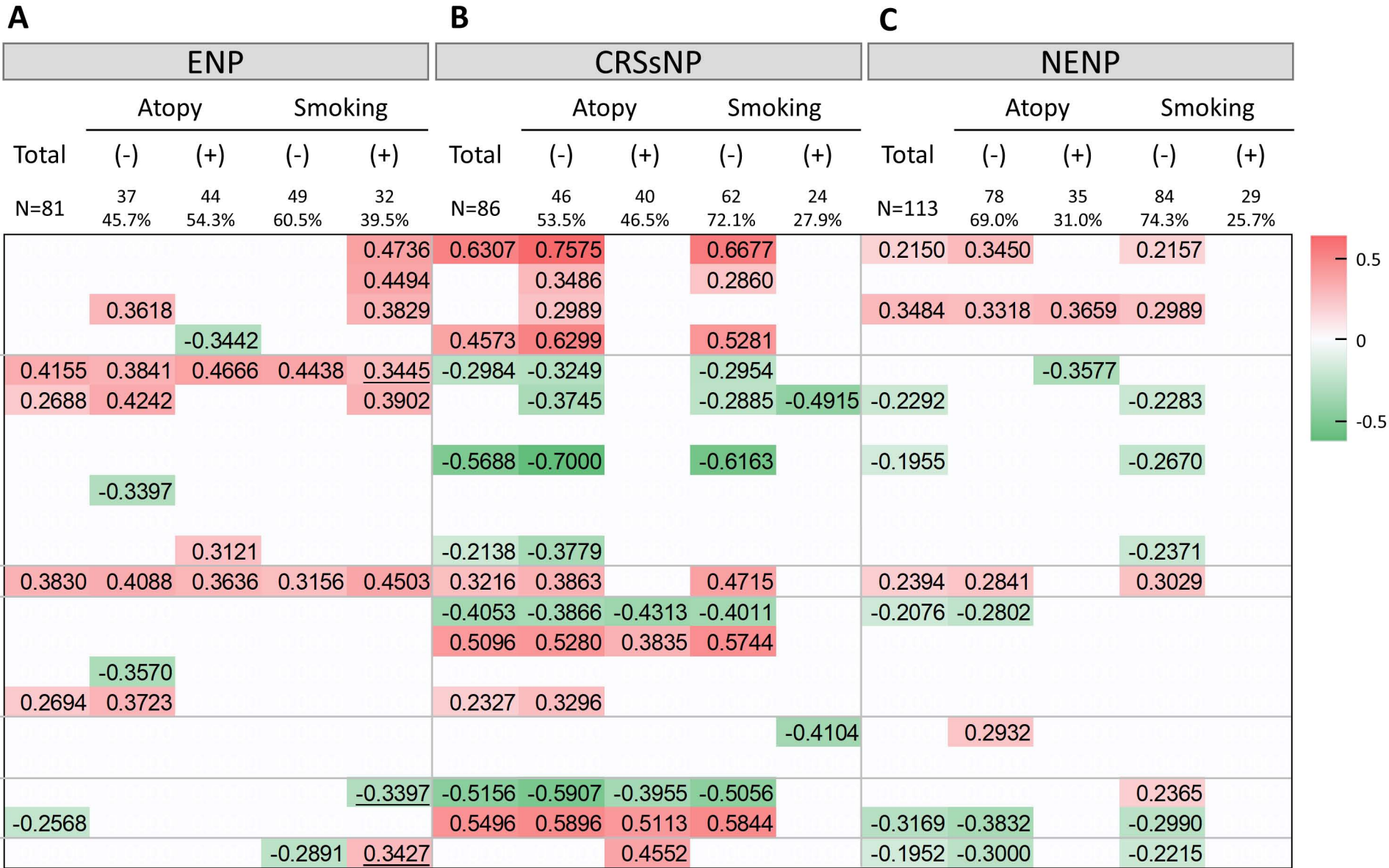
CRSsNP, chronic rhinosinusitis without nasal polyp; *ENP*, eosinophilic nasal polyp; *NA*, not available; *NENP*, non-eosinophilic nasal polyp

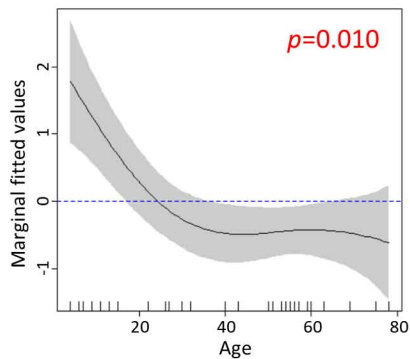
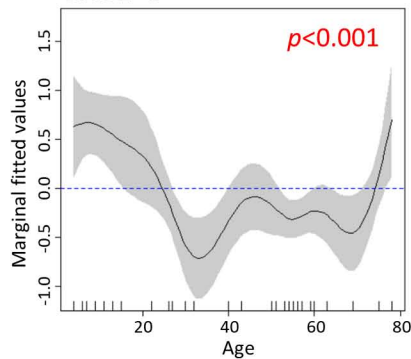
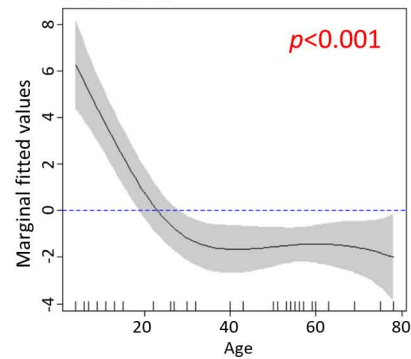
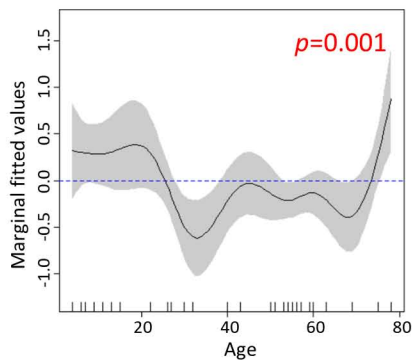
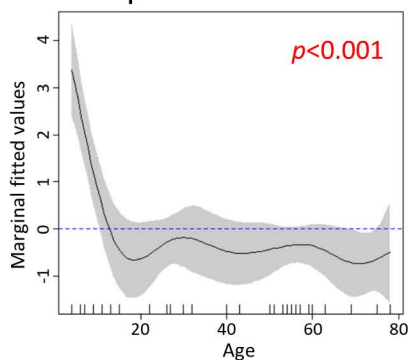
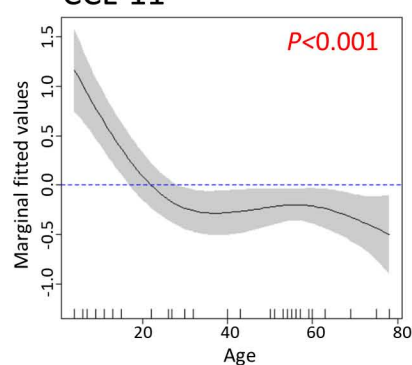
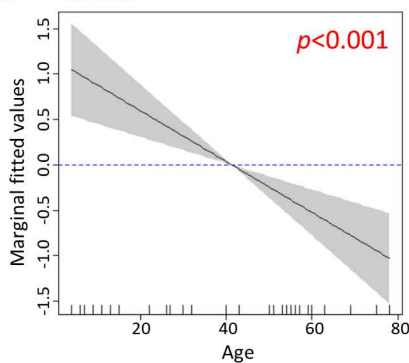
A

		Control	CRS total	CRSsNP	NENP	ENP
		N=29	N=280	N=86	N=113	N=81
Type 2	IL-5		0.3756	0.6307	0.2150	
	CCL-11	-0.6140	0.1656			
	CCL-24		0.2909		0.3484	
	Periostin	-0.5046	0.2528	0.4573		
Type 3	IL-17A	-0.4238	-0.2346	-0.2984		0.4155
	CXCL-1		-0.1863		-0.2292	0.2688
	CXCL-2					
	CXCL-8	-0.5243	-0.3324	-0.5688	-0.1955	
	IL-22					
	IL-23					
	IL-6	-0.4167	-0.1179	-0.2138		
Type 1	IFN- γ		0.2390	0.3216	0.2394	0.3830
Proinflammatory	S100A8	-0.6704	-0.2429	-0.4053	-0.2076	
	IL-1 α		0.1230	0.5096		
	IL-1 β					
	IL-10			0.2327		0.2694
Epithelial-derived	IL-33	-0.5663				
	IL-25	-0.4790				
Others	TGF- β 1	-0.7260	-0.2470	-0.5156		
	BAFF	0.4815		0.5496	-0.3169	-0.2568
CRS severity	LM-CT				-0.1952	

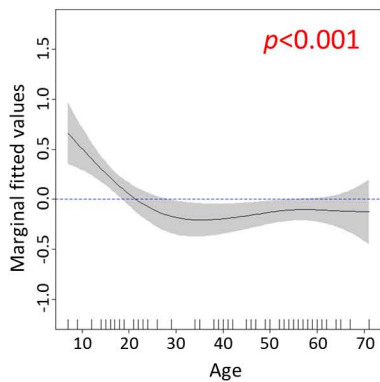
B



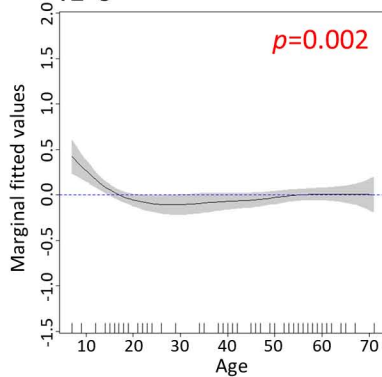


A**IL-17A****CXCL-8****S100A8****IL-6****TGF- β 1****CCL-11****B****IL-33**

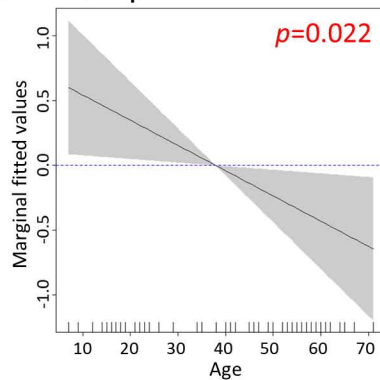
A CXCL-8



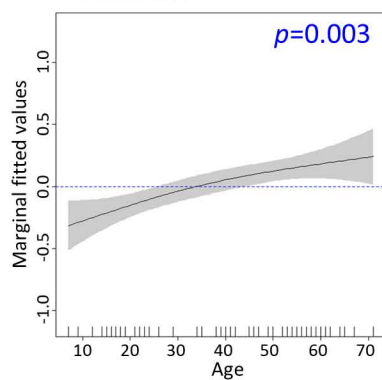
IL-6



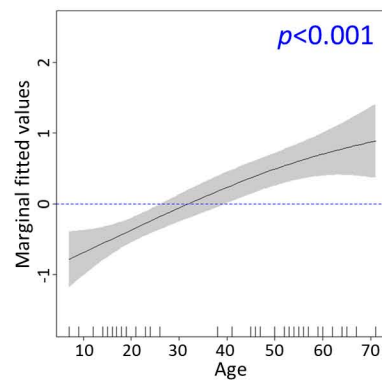
B TGF- β 1



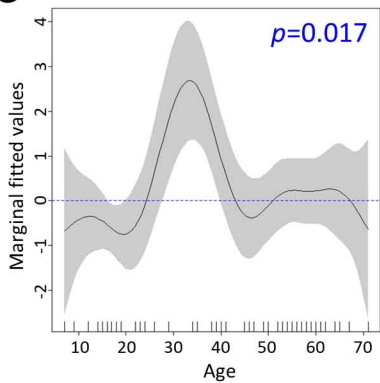
Periostin



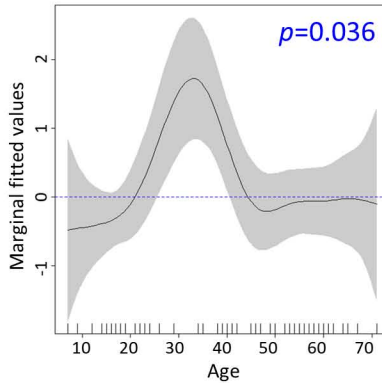
BAFF



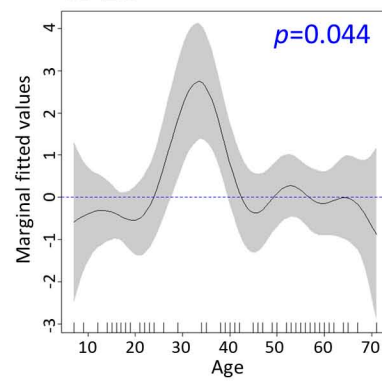
C IL-1 α



IL-25

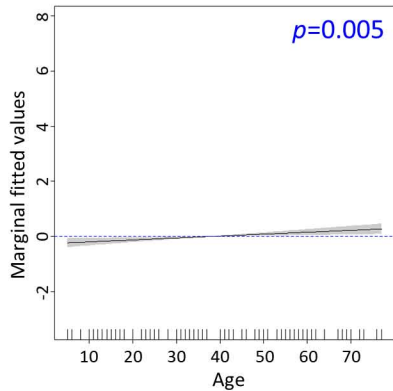
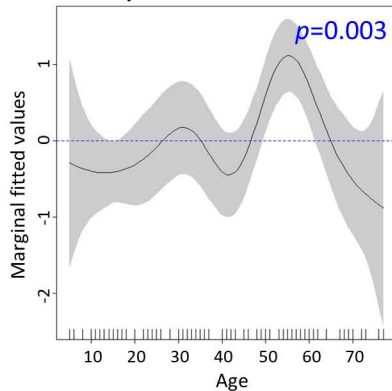


IL-10

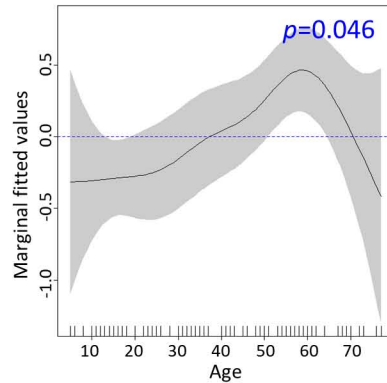


A

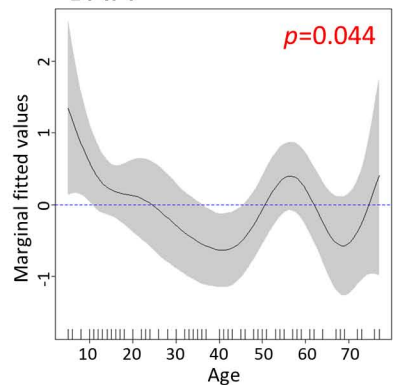
CCL-24

IFN- γ 

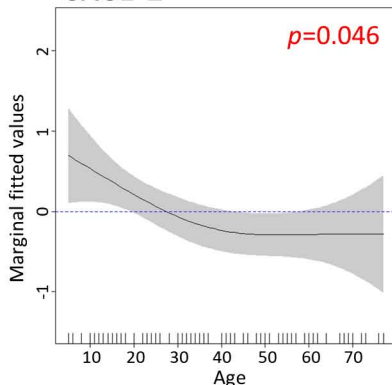
IL-33



BAFF

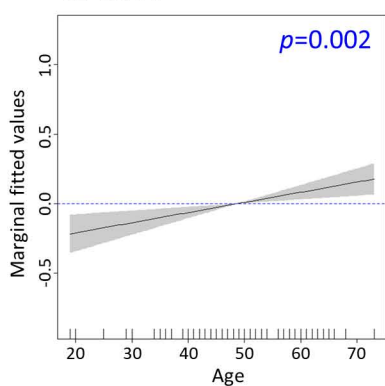


CXCL-1

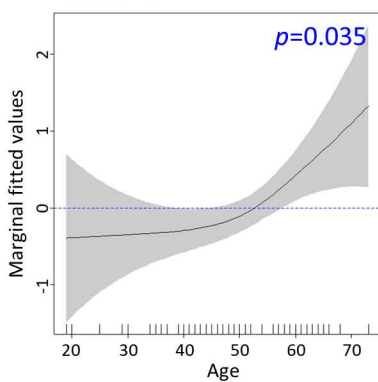


B

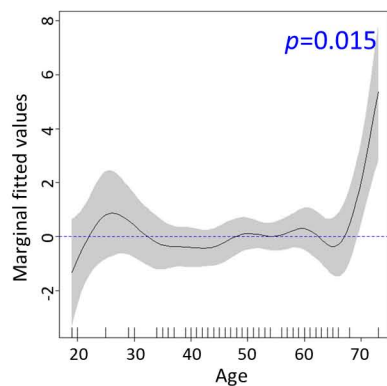
IL-17A



CXCL-1



CCL-24



Frequency (N)

Control
CRSsNP
NENP
ENP

40
30
20
10
0

<10

10-19

20-29

30-39

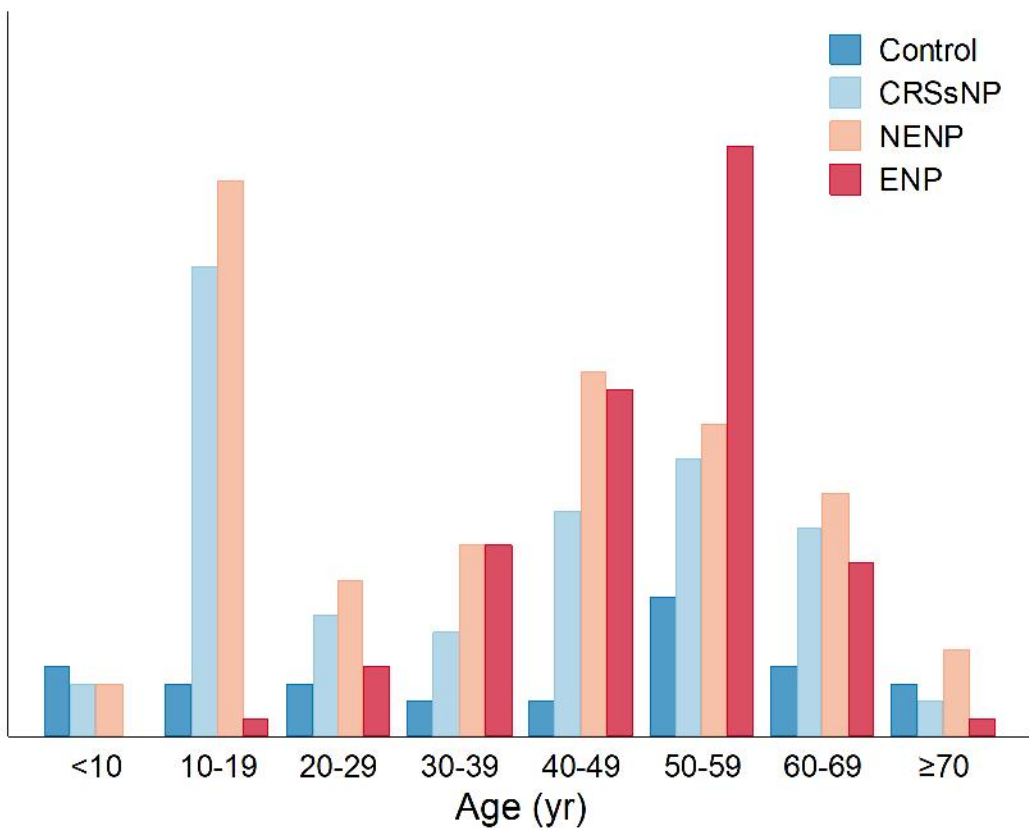
40-49

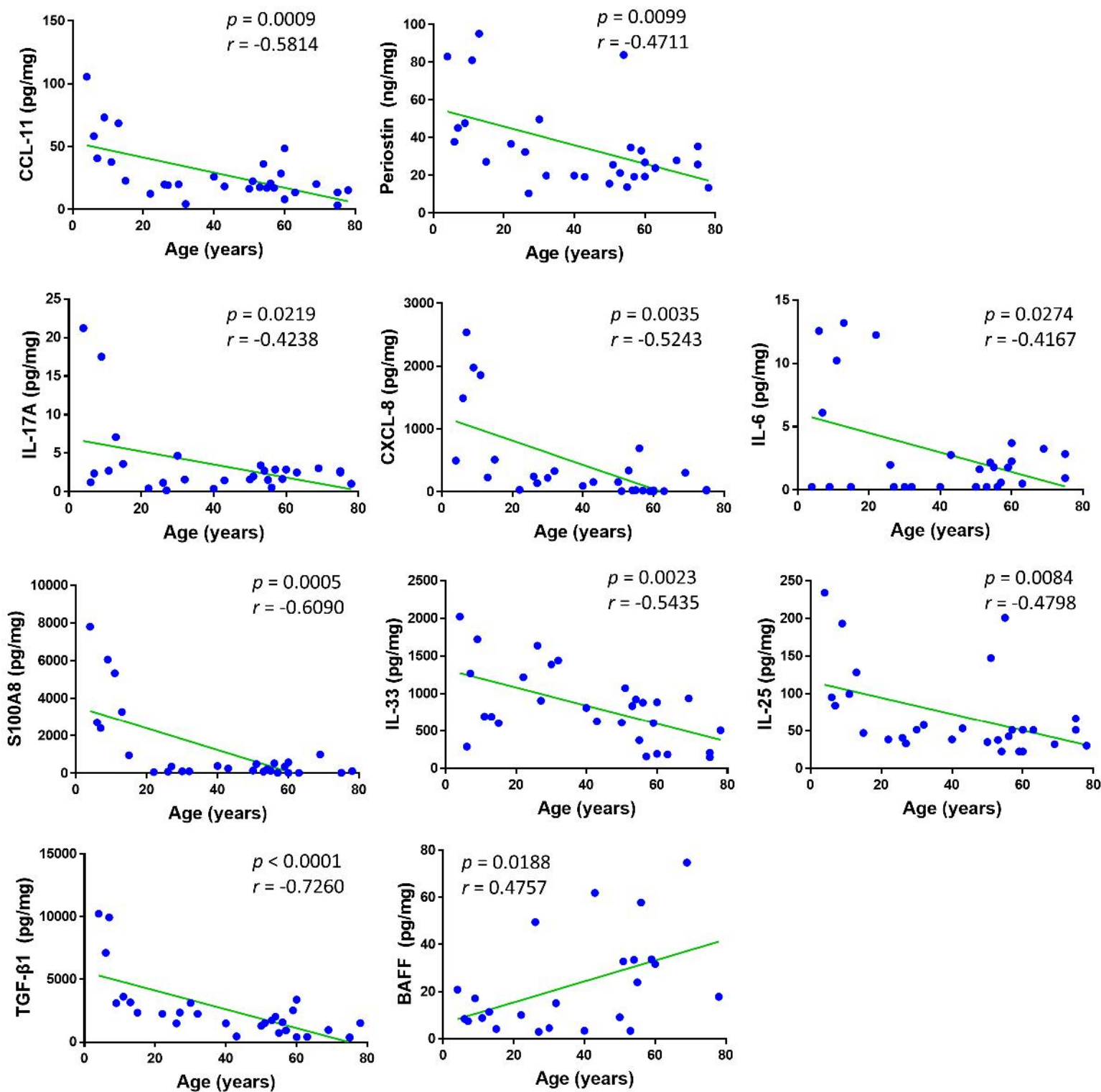
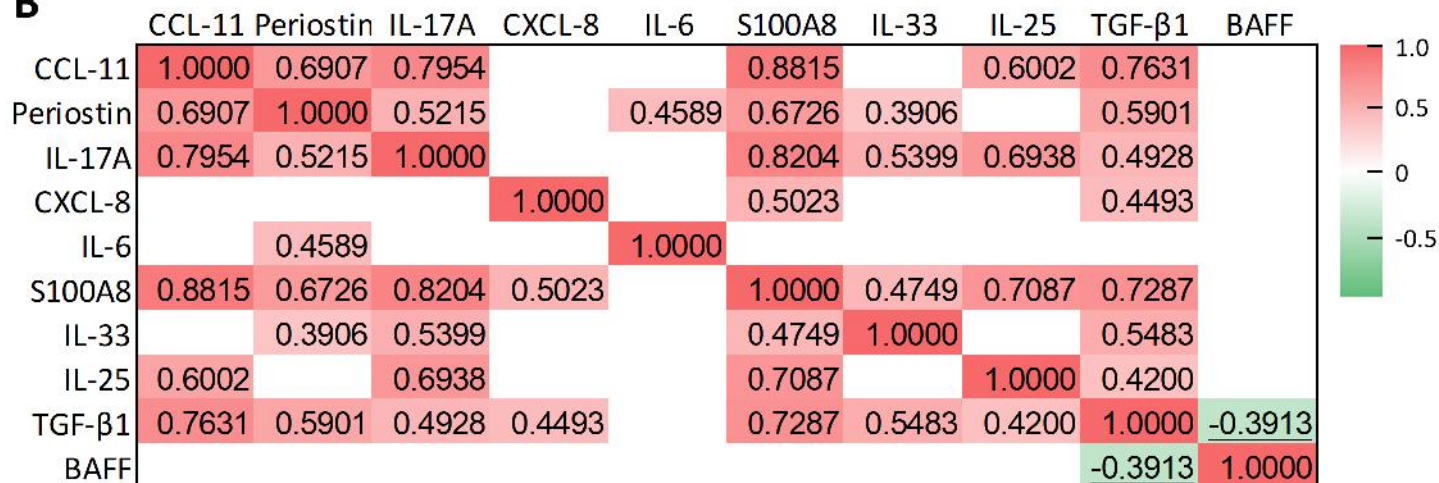
50-59

60-69

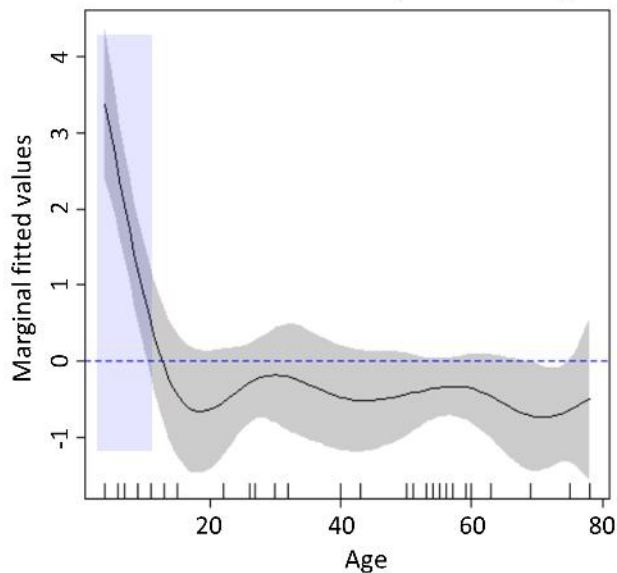
≥70

Age (yr)

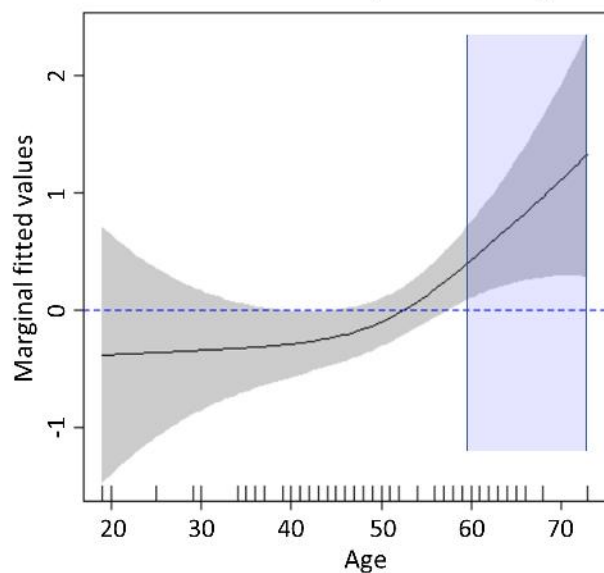


A**B**

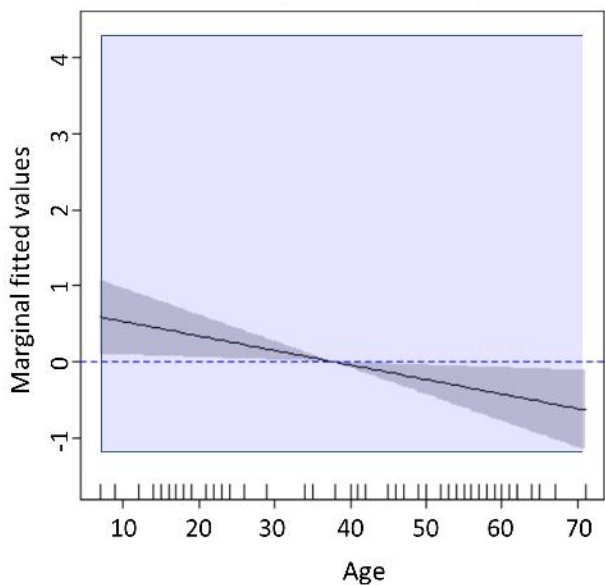
Pattern under 20 years of age



Pattern over 60 years of age



Continuously changing pattern



Specific age-related pattern

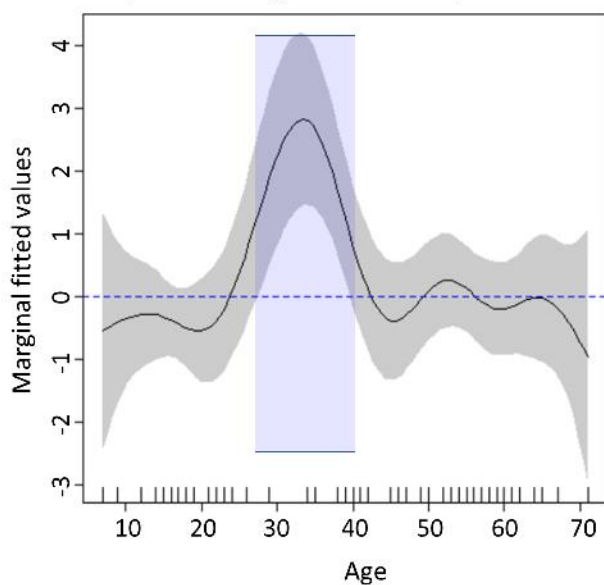


Table S1. Demographics of subtypes of chronic rhinosinusitis

Total no. of subjects	Control (N=29)	CRSsNP (N=86)	NENP (N=113)	ENP (N=81)	P value
Male sex	19 (65.5%)	66 (76.7%)	83 (73.4%)	62 (76.5%)	0.624
Age (y)	50 (22-59)	39.5 (18-54)	40 (16-55)	50 (42-56)	0.009*
Atopic	10 (34.5%)	40 (46.5%)	35 (31.0%)	44 (54.3%)	0.007*
Asthmatic	0	3 (3.5%)	4 (3.5%)	8 (9.9%)	0.242
Smoking	4 (13.8%)	24 (27.9%)	29 (25.7%)	32 (39.5%)	0.043*
Lund-Kennedy score	NA	4 (3-6)	8 (6-10)	6 (4-8)	< 0.001*
Lund-Mackay score	NA	8 (6-13)	13 (10-16)	12 (9-14)	< 0.001*
Blood eosinophil (%)	3.65 (1.6-5)	2.8 (1.6-3.7)	2.6 (1.5-4.4)	5.1 (3.9-6.9)	< 0.001*
Disease duration (month)	NA	60 (12.3-127.1)	60.4 (12.7-122)	60.9 (25.6-114.5)	0.912
SNOT-22	NA	26.5 (14-41)	33 (21-45)	33 (21-45)	0.294

Data are expressed as medians and interquartile ranges or numbers and percentages.

* A *P* value less than 0.05 was considered statistically significant for all analyses

CRSsNP, chronic rhinosinusitis without nasal polyp; *ENP*, eosinophilic nasal polyp; *NA*, not available; *NENP*, non-eosinophilic nasal polyp;

SNOT-22, the 22-item SinoNasal Outcome Test