

Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy

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Background: Nasal polyps (NPs) are characterized by eosinophilic inflammation, which is generally considered sensitive to corticosteroid treatment.

Objectives: We evaluated levels of neutrophilia in NPs and investigated whether increased neutrophilia in polyp tissue affected the response to corticosteroid treatment.

Methods: We studied 3 independent cross-sectional groups of patients with NPs. Levels of infiltration by different types of inflammatory cells were determined by using immunohistochemical analyses and compared with those seen in control nasal tissues from subjects without NPs. Levels of inflammatory mediators were measured by using real-time PCR, ELISA, and FlowCytomix analyses. Patients with NPs received oral corticosteroid therapy (30 mg of prednisone once daily for 7 days); clinical parameters of efficacy were associated with NP phenotypes.

Results: Among patients with NPs, 76.5% had an eosinophilic phenotype, 46.0% had a neutrophilic phenotype, and 35.8% had a mixed phenotype (indicated by double staining). Overall, patients' symptoms improved after corticosteroid treatment; numbers of eosinophils and levels of their mediators (IL-4 and IL-5), but not numbers of neutrophils or levels of their

mediators (IL-8 and interferon-inducible protein 10), were reduced ($P < .05$). After corticosteroid treatment, patients with the nonneutrophilic phenotype (neutrophil negative) had significantly greater reductions in bilateral polyp size scores, nasal congestion scores, total nasal symptom scores, and nasal resistance than patients with the neutrophilic phenotype (neutrophil positive, $P < .05$).

Conclusions: There are different phenotypes of NPs based on the type of immune cell infiltrate and cytokines produced (eosinophilic or neutrophilic). Patients with the neutrophilic phenotype have less response to treatment with corticosteroids based on symptom scores. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Nose, inflammatory response, prognostic factor, response to therapy

Nasal polyps (NPs) have been characterized in histologic studies by excessive expression of T_H2 cytokines and subsequent accumulation of eosinophils but not neutrophils.¹ However, eosinophils might not be the unique effector required for pathogenesis of NPs; NPs have significant heterogeneity among different environments.^{2,3} Significant numbers of neutrophils and T_H17 cells, as well as eosinophils and plasma cells, accumulate in the nasal mucosa of patients with NPs.⁴⁻⁶ Furthermore, levels of IL-8, a chemokine that induces chemotaxis of neutrophils, are 5-fold higher in turbinates of patients with NPs than in healthy control subjects.⁷ However, little is known about the effects of neutrophil infiltration on the pathogenesis and treatment of NPs.

We investigated whether accumulation of neutrophils in NP tissues has the same effects as seen in lung tissues of patients with asthma. Neutrophil infiltration is common in patients with persistent and difficult-to-treat asthma, although asthma was considered an eosinophilic disease, with inflammation induced through a T_H2 -type response.⁸ Although most patients with mild-to-moderate asthma respond well to inhaled corticosteroids, a few patients with severe asthma, as characterized by neutrophil recruitment, are largely unresponsive to treatment with systemic corticosteroids.⁹ Corticosteroids are the most effective treatment to reduce NP size and improve nasal symptoms.^{10,11} However, it is not clear whether increased infiltration of polyp tissues by neutrophils affects the clinical response to oral corticosteroids.

We studied a large cohort of patients with NPs to evaluate the recruitment of eosinophils and neutrophils to polyp tissues and to determine whether NPs with increased numbers of neutrophils indicate different levels of sensitivity to corticosteroid treatment.

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Abbreviations used

ANCOVA: Analysis of covariance
 Ct: Cycle threshold
 HNE: Human neutrophil elastase
 hpfs: High-power field
 IP-10: Interferon-inducible protein 10
 IQR: Interquartile range
 MBP: Major basic protein
 MPO: Myeloperoxidase
 NP: Nasal polyp
 TNSS: Total nasal symptom score

METHODS**Study design and subjects**

Subjects with NPs were recruited from participating hospitals; they received their diagnoses based on the standard criteria for NPs issued in the European Position Paper on Rhinosinusitis and Nasal Polyps 2007 guidelines.¹ The control group comprised patients undergoing septoplasty for anatomic variations. Tissues were removed from the inferior turbinate during septal surgery. None of the subjects used oral or nasal corticosteroids or other medications (eg, antibiotics or antileukotrienes) for 4 weeks before sample collection. All subjects were free from upper respiratory tract infections for the 4 weeks preceding the study.

The Lund-Kennedy scoring system (0–6) was used to grade the severity of NPs, as follows: 0, no polyps; 1, polyps in the middle meatus but not reaching below the inferior border of the middle turbinate; 2, polyps reaching below the inferior border of the middle turbinate but not to the inferior border of the inferior turbinate; and 3, extensive large polyps congesting the inferior meatus. Individual nasal symptom scores included nasal congestion, anterior rhinorrhea, postnasal drip, and loss of smell. They were evaluated by a physician and assigned scores of 0 to 3, as follows: 0, no symptoms; 1, mild symptoms occasionally present; 2, moderate symptoms frequently present; and 3, severe symptoms continuously present. Total nasal symptom scores (TNSSs) were then calculated (range, 0–12). Atopic status was evaluated by using a skin prick test or by using assays for specific IgEs (Phadia, Uppsala, Sweden) against common inhaled allergens (eg, pollens, dust mites, pets, molds, and cockroaches). Specific IgE values of 0.35 kIU/L or greater were considered positive. Nasal resistance was tested with the RhinoMetrics SRE2100 (Interacoustics A/S, Assens, Denmark), according to the manufacturer's instructions. The diagnosis of asthma was determined by a pulmonologist.

Subjects were assigned to 1 of 3 independent cross-sectional groups. Their sociodemographic and clinical characteristics, including polyp size and nasal symptoms, are presented in **Tables I and II**. In brief, group 1 included 187 adult patients with NPs and 45 control subjects; histologic samples were reviewed. Group 2 included 31 patients with NPs and 17 control subjects; correlations between neutrophil infiltration and levels of inflammatory mediators were determined. Group 3 included 48 patients with NPs; the therapeutic response to oral corticosteroids (30 mg of prednisone once daily for 7 days) was evaluated. The primary and secondary efficacy end points are described in the **Methods** section in this article's Online Repository at www.jacionline.org. This study was approved by the local ethics committees of the participating hospitals, and informed consent was obtained from each subject.

Immunohistochemical analyses

Human nasal tissues were analyzed by means of immunohistochemistry to determine infiltration by different inflammatory cells. The following mAbs were used: anti-CD3 (1:100; Dako, Glostrup, Denmark), anti-CD20 (1:200, Dako), anti-major basic protein (MBP; 1:50, Dako), anti-human neutrophil elastase (HNE; 1:50; Serotec, Oxford, United Kingdom), anti-IL-17 (1:100; R&D Systems, Minneapolis, Minn), and anti-tryptase (1:200, Dako). The colocalization of eosinophils and neutrophils was examined with a Multi-Vision Polymer Detection System (Thermo Fisher Scientific, Fremont,

Calif), according to the manufacturer's instructions. Two independent observers blind to the diagnoses and clinical data counted the number of positive cells in 10 randomly selected high-power fields (hpfs; $\times 400$ magnification), and average values were calculated. The detailed method for immunohistochemical analysis is described in the **Methods** section in this article's Online Repository.

The cutoff values for the eosinophil-positive, eosinophil-negative, neutrophil-positive, and neutrophil-negative phenotypes were set based on histologic data. The cutoff value for the eosinophil-positive phenotype was set at 8 MBP⁺ cells/hpf, which is equivalent to the mean value in the healthy control subjects (2.48) plus 2 times the SD ($2.54 \times 2 = 5.08$; cutoff value, $2.48 + 5.08 = 7.56$). The cutoff value for the neutrophil-positive phenotype was set at 4 HNE⁺ cells/10 hpfs, which was calculated as the mean value in the healthy control subjects (1.23) plus 2 times the SD ($1.35 \times 2 = 2.70$; cutoff value, $1.23 + 2.70 = 3.93$).

On the basis of the immunohistochemical findings, NPs with more than 8 HNE⁺ cells per hpf (twice the cutoff value of the neutrophil-positive phenotype) were defined as having a neutrophil-high phenotype. NPs with more than 15 MBP⁺ cells per hpf (nearly 2-fold the cutoff value of the eosinophil-positive phenotype) were defined as having the eosinophil-high phenotype.

Quantitative RT-PCR

The mRNA levels of select chemokines (*CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10*) were determined by using quantitative RT-PCR. The primer sequences are listed in **Table E1** in this article's Online Repository at www.jacionline.org. The methods are described in the **Methods** section of this article's Online Repository.

ELISA

The levels of myeloperoxidase (MPO), IL-8, and interferon-inducible protein 10 (IP-10) in nasal homogenates are measured by ELISA. The details are described in the **Methods** section in this article's Online Repository.

FlowCytomix analysis

The details for nasal secretion collection and FlowCytomix analysis (eBioscience, San Diego, Calif) of IFN- γ , IL-4, IL-5, IL-8, and IL-17 are described in the **Methods** section in this article's Online Repository.

Statistical analysis

Categorical variables are expressed as medians and interquartile ranges (IQRs). Continuous variables are expressed as means and SEs of the mean. The statistical analysis was performed by using the Mann-Whitney *U* test for unpaired comparisons. For efficacy evaluation, baseline variables were analyzed with 1-way ANOVA followed by the paired Student *t* test for differences before and after treatment. Comparisons between the 3 patient groups were performed by using an analysis of covariance (ANCOVA) model, and the least-square means were obtained from ANCOVA with age of patients, duration of symptoms, eosinophil numbers in polyp tissues, baseline values, atopy, and asthma status. The Spearman rank correlation test was used to analyze the correlations among different parameters. A *P* value of less than .05 was considered significant.

RESULTS**Eosinophil and neutrophil infiltration of NP and control tissues**

The clinical characteristics of group 1 are shown in **Table I**. The presence of eosinophils and neutrophils in nasal tissues was determined by means of immunohistochemical staining for MBP and HNE. Representative results for eosinophils, neutrophils, and IL-17⁺ cells are presented in **Fig E1** in this article's Online Repository at www.jacionline.org.

TABLE I. Clinical characteristics of patients with NPs and control subjects (groups 1 and 2)

Characteristic	Group 1		Group 2	
	Patients with NPs	Control subjects	Patients with NPs	Control subjects
No.	187	45	31	17
Age (y), mean (range)	35.4 (21-54)	26.5 (22-34)	33.5 (22-45)	23.2 (21-29)
Sex (male/female)	115/72	29/18	19/12	10/7
Duration (y), mean (range)	5.2 (2-14)	0	4.5 (1-9)	0
Atopic	73	0	12	0
Nonatopic	114	45	19	17
Asthma in history	16	0	2	0
Aspirin intolerance	0	0	0	0

TABLE II. Clinical characteristics of patients with NPs (group 3)

Characteristic	Patients with NPs	
	Baseline	After treatment (day 7)
No.	45	
Age (y), mean (range)	31.9 (24-51)	
Sex (male/female)	25/20	
Duration (y), mean (range)	4.7 (3-11)	
Atopic	4	
Nonatopic	41	
Asthma in history	2	
Aspirin intolerance	0	
Polyp size, mean (SEM)	5.25 (0.52)	4.17 (0.90)*
Nasal congestion, mean (SEM)	2.79 (0.39)	1.90 (0.75)*
Anterior rhinorrhea, mean (SEM)	2.64 (0.44)	2.12 (0.59)*
Postnasal drip, mean (SEM)	2.58 (0.45)	2.05 (0.58)*
Loss of smell, mean (SEM)	2.94 (0.27)	1.76 (0.67)*
TNSS, mean (SEM)	10.95(1.50)	7.83 (2.47)*
Nasal resistance (Pa.s/cm ³), mean (SEM)	1.46 (0.28)	0.93 (0.45)*

* $P < .05$ compared with baseline values.

Eosinophils and neutrophils were each frequently detected in polyp tissues. The lamina propria in polyp tissues had strong and extensive staining for MBP and HNE, whereas the epithelial and glandular areas had less staining. The median numbers of eosinophils per hpf in the polyp and control tissues were 11.5 (IQR, 8.2-15.8) and 1.6 (IQR, 0.6-3.6), respectively, and the median numbers of neutrophils per hpf in the polyp and control tissues were 3.5 (IQR, 1.7-7.5) and 0.8 (IQR, 0.3-1.35), respectively ($P < .05$, Fig 1). We also found large numbers of IL-17⁺ cells in whole polyp tissues. The median numbers of IL-17⁺ cells per hpf in the polyp and healthy control tissues were 10.2 (IQR, 4.6-16.4) and 1.2 (IQR, 0.5-3.1), respectively ($P < .05$). Numbers of eosinophils and neutrophils correlated with the number of IL-17⁺ cells in polyp tissues (eosinophils vs IL-17⁺ cells, $r = 0.55$, $P < .05$; neutrophils vs IL-17⁺ cells, $r = 0.82$, $P < .05$). In addition, the numbers of mast cells (tryptase positive), B cells (CD20⁺), and T cells (CD3⁺) were significantly increased in polyp tissues compared with those seen in control tissues ($P < .05$, data not shown).

On the basis of defined cutoff values, 76.5% of the NPs had the eosinophil-positive phenotype, and 46.0% had the neutrophil-positive phenotype. These percentages were significantly greater than in the control nasal tissues (6.7% eosinophil positive and 6.7% neutrophil positive, $P < .05$). A mixed phenotype (eosinophil positive and neutrophil positive) was observed in 35.8% of

the NP samples based on double staining; this value was significantly greater than in control tissues (2.2%, $P < .05$). The neutrophil-positive NPs had a significantly higher frequency of B-cell and eosinophil infiltration than the neutrophil-negative NPs ($P < .05$) but no differences in T-cell or mast cell infiltration (data not shown). We observed significantly higher levels of eosinophils, but not neutrophils, in patients with larger polyps, atopy, and comorbid asthma (see Fig E2 in this article's Online Repository at www.jacionline.org).

Levels of neutrophil-associated inflammatory mediators in NP and control tissues

The clinical characteristics of group 2 are shown in Table II. The mRNA levels of neutrophil-associated chemokines (*CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10*) in nasal tissues were examined by using real-time PCR; we specifically analyzed mRNA levels in NPs with neutrophil infiltration. These mRNAs were detectable in all samples analyzed; levels of *CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10* mRNAs were significantly increased in NP tissues compared with those seen in control tissues ($P < .05$, see Fig E3 in this article's Online Repository at www.jacionline.org). Levels of *CXCL5*, *CXCL8*, and *CXCL10* mRNA correlated with and neutrophil infiltration of NP tissues (*CXCL5* vs neutrophils, $r = 0.62$, $P < .05$; *CXCL8* vs neutrophils, $r = 0.87$, $P < .05$; *CXCL10* vs neutrophils, $r = 0.58$, $P < .05$). Levels of *CXCL5*, *CXCL8*, and *CXCL10* mRNA were significantly higher in the neutrophil-positive NPs than in the neutrophil-negative NPs ($P < .05$, data not shown).

We analyzed levels of MPO, IL-8, and IP-10 in nasal tissue homogenates from group 2 by using ELISA. The levels of MPO, IL-8, and IP-10 were significantly increased in NPs compared with those seen in healthy tissues ($P < .05$, Fig 2). When neutrophil-negative and neutrophil-positive NPs were compared, the levels of MPO, IL-8, and IP-10 were significantly higher in the neutrophil-positive NPs ($P < .05$).

Changes in clinical parameters and cytokine levels after prednisone therapy

The clinical characteristics of group 3 are shown in Table II. After 1 week of treatment with oral prednisone, the polyp size score, nasal resistance, nasal congestion score, and TNSS were significantly reduced compared with baseline values ($P < .05$). The levels of eosinophils, but not neutrophils, were also significantly decreased in NP tissues after corticosteroid treatment. Levels of IFN- γ , IL-4, and IL-5 (but not IL-8 or IL-17) were significantly reduced in nasal secretions after treatment (Fig 3).

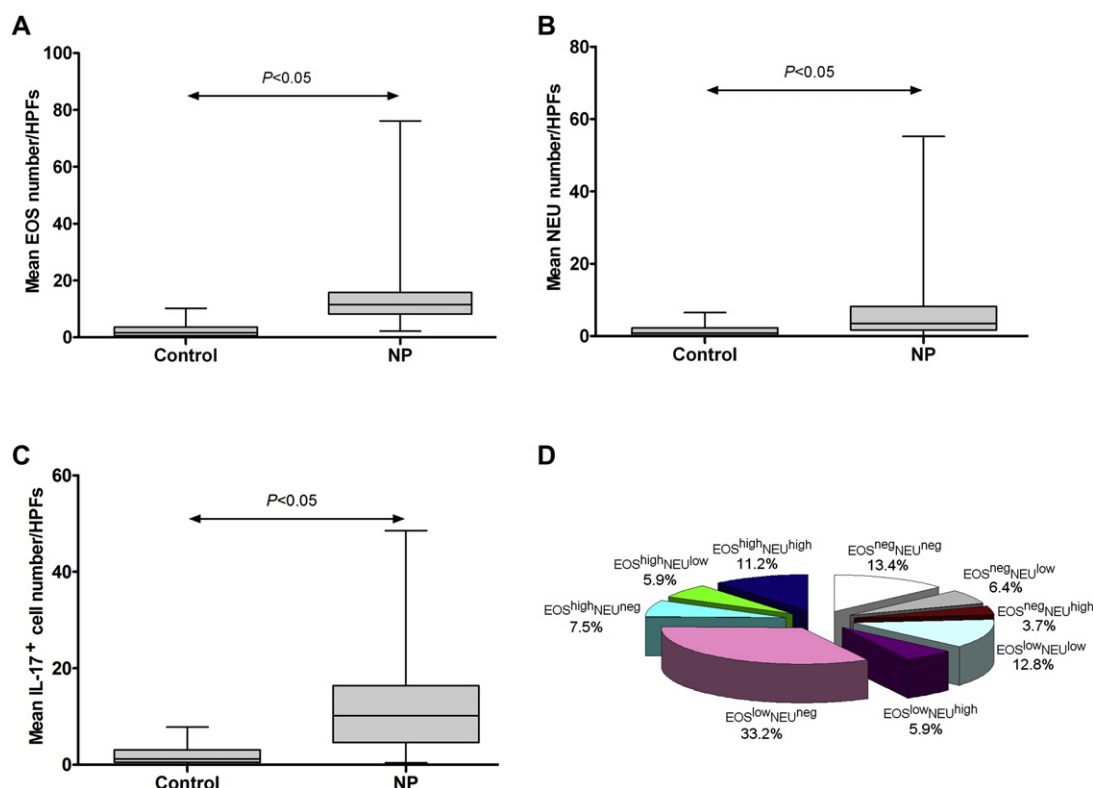


FIG 1. Numbers of eosinophils (EOS), neutrophils (NEU), and IL-17⁺ cells in NP and control tissues. **A-C**, On the basis of immunohistochemical analysis, levels of eosinophils, neutrophils, and IL-17⁺ cells were significantly higher in NP than control tissues. **D**, The different proportions of eosinophils and neutrophils in NP tissues are also presented. Data are represented by box-and-whisker plots; the Mann-Whitney *U* test was used for statistical analysis.

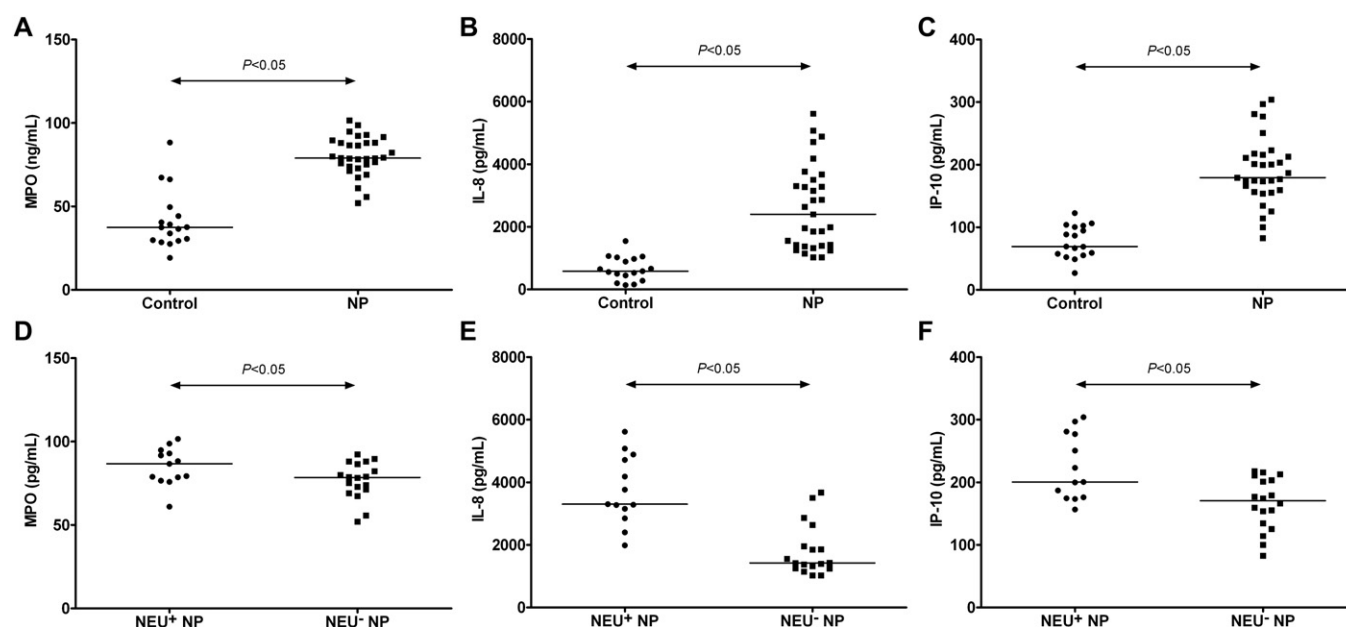


FIG 2. Protein levels of MPO, IL-8, and IP-10 in NP and control tissues. **A-C**, Levels of MPO (Fig 2, A), IL-8 (Fig 2, B), and IP-10 (Fig 2, C) in NP tissues were significantly higher than in control tissues. **D-F**, When subdivided into neutrophil-negative and neutrophil-positive phenotypes, levels of MPO (Fig 2, D), IL-8 (Fig 2, E), and IP-10 (Fig 2, F) were significantly higher in neutrophil-positive than in neutrophil-negative NPs. Data are represented by a box-and-whisker plot. Solid bars represent median values; the Mann-Whitney *U* test was used for statistical analysis.

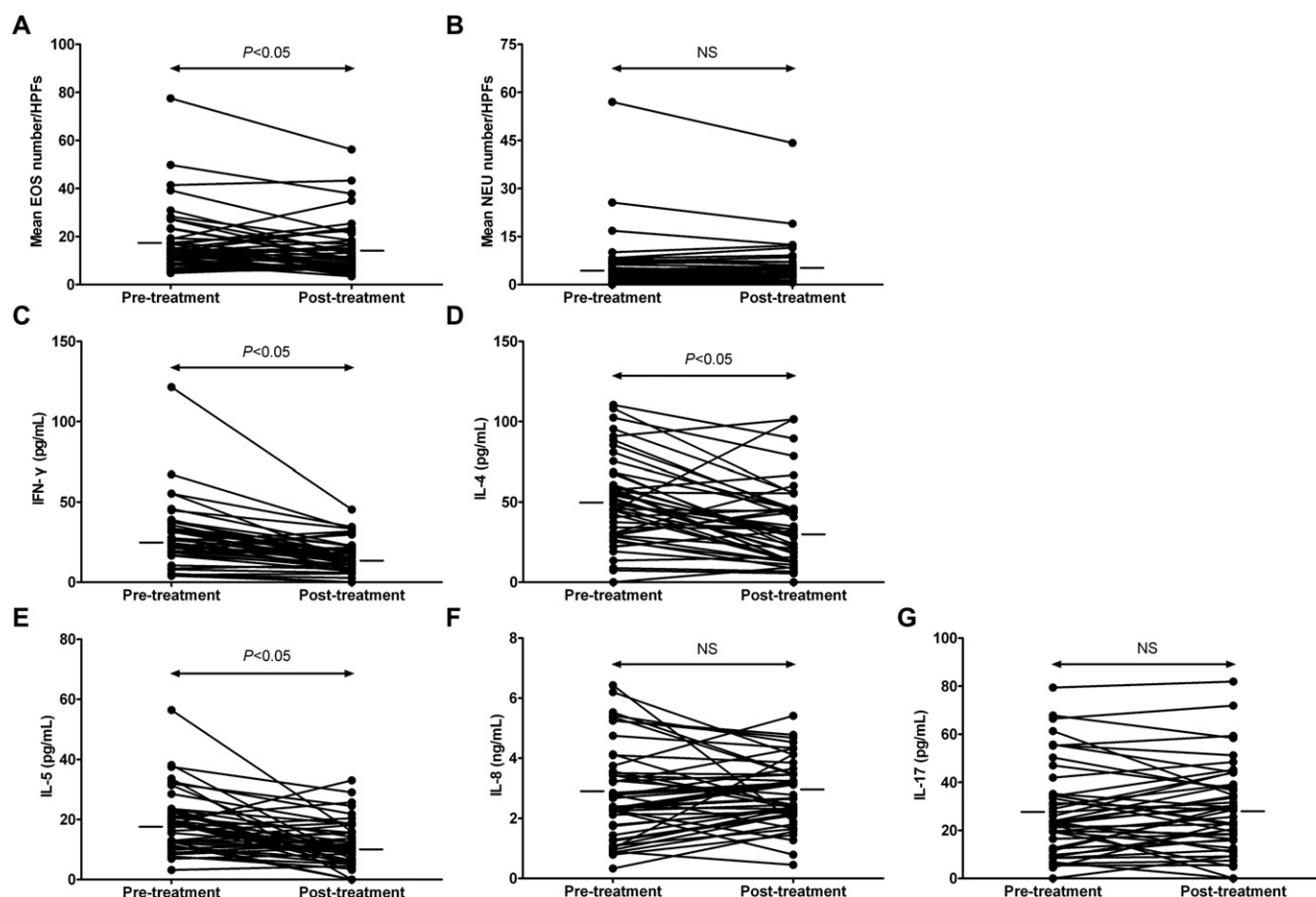


FIG 3. Changes from baseline in clinical parameters and cytokine levels in patients treated with oral prednisone. **A** and **B**, After 1 week of treatment with oral prednisone, levels of eosinophils (Fig 3, **A**), but not neutrophils (Fig 3, **B**), were significantly reduced in NP tissues. **C-G**, Levels of IFN- γ (Fig 3, **C**), IL-4 (Fig 3, **D**), and IL-5 (Fig 3, **E**), but not IL-8 (Fig 3, **F**) and IL-17 (Fig 3, **G**), were significantly decreased in nasal secretions after treatment. Solid bars represent mean values; the paired Student *t* test was used for statistical analysis. NS, Not significant.

When patients with neutrophil-negative NPs and neutrophil-positive NPs were compared, we observed significantly smaller reductions in the bilateral polyp size scores on day 7 in the neutrophil-low (1.20 points) and neutrophil-high groups (0.39 points) compared with those seen in the neutrophil-negative group (1.69 points; $P < .05$; Fig 4, **A**). Similarly, significantly smaller decreases in nasal congestion scores (neutrophil low, 0.78 points; neutrophil high, 0.43 points; neutrophil negative, 1.52 points), TNSSs (neutrophil low, 3.05 points; neutrophil high, 1.52 points; neutrophil negative, 4.93 points), and nasal resistance (neutrophil low, 0.44 points; neutrophil high, 0.35 points; neutrophil negative, 0.91 points) were observed in patients with neutrophil-negative versus eosinophil-positive NPs ($P < .05$; Fig 4, **B-D**). However, there were no significant differences in reductions of scores for rhinorrhea, postnasal drip, or loss of smell between patients with neutrophil-negative NPs and neutrophil-positive NPs (data not shown).

DISCUSSION

In the present study we demonstrated that NPs have higher levels of neutrophil infiltration and neutrophil-related mediators than

nasal tissues from control subjects. We can define different phenotypes of NPs based on cell composition and levels of neutrophil-associated mediators. NPs with these different phenotypes (neutrophil negative and neutrophil positive) have different levels of response to prednisone therapy. Polyp histology might therefore be used to predict the response to corticosteroid treatment.

NPs are believed to be a multifactorial and heterogeneous disease; they are frequently associated with asthma and other respiratory diseases, including cystic fibrosis and aspirin sensitivity. Previous histologic analyses of NP infiltrates reported that 80% of polyps have an abundance of eosinophils.^{12,13} We also detected large numbers of eosinophils, along with mast cells, B cells, and T cells, in NP tissues. In this study 76.5% of NPs had increased eosinophil infiltration compared with that seen in control tissues; numbers of eosinophils were significantly associated with NP size, atopy, and comorbid asthma. Our findings, together with our previous report on levels of IL-5 in NP tissues,¹⁴ support the hypothesis that eosinophilic inflammation is a common and basic pathophysiologic process.

On the basis of the observed eosinophil infiltration, NPs have generally been classified into eosinophilic and noneosinophilic phenotypes.^{6,15} However, this classification system ignores the

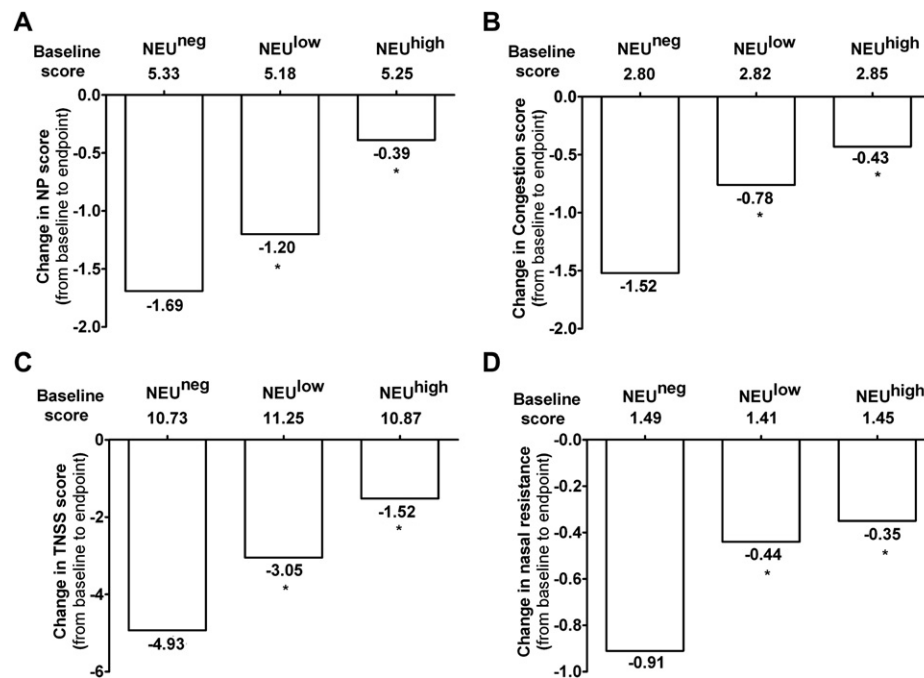


FIG 4. Changes from baseline in NP size, TNSS, nasal congestion score, and nasal resistance after oral prednisone treatment. **A**, Patients with neutrophil (*NEU*)^{low} and neutrophil-high NPs had significantly smaller decreases in bilateral polyp size scores than patients with neutrophil-negative NPs. **B–D**, Patients with neutrophil-low and neutrophil-high NPs also had significantly smaller decreases in nasal congestion scores (Fig 4, *B*), TNSSs (Fig 4, *C*), and nasal resistance (Fig 4, *D*). Baseline variables were analyzed with the ANOVA test, followed by a paired Student *t* test for differences before and after treatment, to determine efficacy. Comparisons between the 3 groups were performed with the ANCOVA model; the least-squares means were obtained from ANCOVA with the ages of the patients, duration of symptoms, numbers of eosinophils in polyp tissues, baseline values, atopy, and asthma status. **P* < .05 compared with neutrophil negative.

roles of neutrophils in the pathogenesis of NPs and might overemphasize the importance of eosinophils. In this study we created a new classification system for NPs that includes neutrophil-positive and neutrophil-negative groups based on defined cutoff values. Interestingly, we found that 46.0% of NP samples were of the neutrophil-positive phenotype and 35.8% were of a mixed (eosinophil positive and neutrophil positive) phenotype. Furthermore, numbers of IL-17⁺ cells were significantly increased in polyp tissues compared with those seen in control tissues. Even though IL-17 increases production of IL-8 and infiltration by neutrophils, the number of IL-17⁺ cells correlated not only with numbers of neutrophils but also numbers of eosinophils in polyp tissues. These findings indicate that NP pathogenesis involves a complex inflammatory process.

In analyzing the expression of neutrophil-associated chemokines, mRNA levels of *CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10* were significantly increased in NP compared with control tissues. Furthermore, levels of *CXCL5*, *CXCL8*, and *CXCL10* mRNA correlated with neutrophil infiltration of NP tissues. These findings were supported by the increased levels of MPO, IL-8, and IP-10 in NP tissue homogenates (measured by means of ELISA). Neutrophil-associated chemokines could therefore mediate accumulation and activation of neutrophils in polyp tissues.

Neutrophils are similar to eosinophils in that they are effectors of host defense against infection by bacteria or parasites. On activation, both cell types perform important biologic functions, such as secreting several types of cytotoxic and degradative enzymes. Neutrophils infiltrate some tissues during allergic or

asthmatic reactions that have been characterized by eosinophilic inflammation. In asthmatic patients neutrophil infiltration of lung tissue has been described as a severe unstable subtype that is resistant to corticosteroid treatment.^{16,17} Fukakusa et al¹⁸ showed that corticosteroids are not capable of reducing neutrophilia or the expression of neutrophil-associated chemokines in the airways of asthmatic patients, although they are effective in reducing eosinophilia and the expression of eosinophil-associated chemoattractants. Neutrophilic polyps that develop in patients with cystic fibrosis are also resistant to corticosteroid treatment.¹⁹ Our observations of neutrophil infiltration of NP tissues could be similar to those observed in lung tissues of patients with asthma and associated with corticosteroid hyporesponsiveness.

We compared the responses of neutrophil-positive and neutrophil-negative NPs to oral prednisone treatment. Systemic and intranasal corticosteroids are currently considered the first-line medical treatment for patients with NPs. Corticosteroids have potent anti-inflammatory actions and can reduce polyp grade (size and extent), improve symptoms, and delay or reduce the need for surgery by inhibiting eosinophil-associated inflammation.²⁰ A double-blind, placebo-controlled study by Hissaria et al²¹ reported that 14 days of therapy with 50 mg of prednisone significantly reduced polyp size and symptoms compared with placebo. However, corticosteroid therapy is not equally effective in all patients.²² Patients who received 7 days of therapy with 30 mg of prednisone had overall reductions in polyp size, symptom scores, nasal resistance, nasal congestion scores, and TNSSs compared with baseline values and in accordance with the previous

studies. As expected, the levels of eosinophils, but not neutrophils, were also significantly decreased in NP tissues after corticosteroid treatment. The effect of corticosteroid treatment on eosinophil accumulation, apoptosis, or both might partially account for its clinical efficacy. On the other hand, corticosteroids did not affect levels of IL-8 and IL-17 or numbers of neutrophils. Neutrophil-mediated inflammation might therefore affect the efficacy of oral corticosteroid therapy for NPs. We divided patients into subgroups based on whether their NPs were neutrophil negative, neutrophil low, or neutrophil high and compared the efficacy of 7 days of oral prednisone treatment. To our knowledge, this was the first study to directly compare the effect of corticosteroids on NPs with different levels of neutrophil infiltration. After treatment, we observed significantly smaller reductions in bilateral polyp size scores, nasal congestion scores, TNSSs, and nasal resistance among patients with neutrophil-low or neutrophil-high NPs compared with neutrophil-negative NPs. NPs that are infiltrated with neutrophils (high or low levels) therefore respond differently to prednisone treatment than polyps that do not contain neutrophil infiltrates.

As a limitation, this study lacked a placebo group, which makes it difficult to measure therapeutic efficacy. Although there was less improvement in neutrophil-positive polyp tissues after corticosteroid treatment, polyps of the neutrophil-high phenotype had some degree of response to prednisone. Data from a placebo group could be used to quantify this response. Also, in this study the dose of prednisone given was lower, and the duration of therapy was shorter than in normal clinical practice. The responses observed might have been greater if patients received higher doses or longer durations of therapy. A placebo-controlled trial is therefore necessary to strengthen the conclusion.

Nonetheless, we have identified different phenotypes of NPs based on the composition of cell infiltrates and levels of immune mediators. We have associated increased neutrophilia in polyp tissues with reduced response to corticosteroid therapy. Reagents that target neutrophil-associated mediators and tissue neutrophilia might improve treatment of NPs in a specific population of patients.

Clinical implications: Oral corticosteroid therapy reduces numbers of eosinophils, but not neutrophils, in NPs. Increased infiltration of NPs by neutrophils reduces the clinical response to oral corticosteroids. Different therapeutic strategies might therefore be necessary for patients with neutrophil-predominant NPs.

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METHODS

Subjects

This study analyzed data from 3 groups of patients with NPs ($n = 187$) enrolled from the participating hospitals. The control group consisted of 47 patients undergoing septoplasty because of anatomic variations, and tissues were isolated from the inferior turbinate during septal surgery. In this study NPs were defined based on endoscopic findings of bilateral polyps originating from the middle meatus and/or confirmation of NPs by using computerized tomography, according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2007 guidelines.

Subjects were recruited into 3 independent cross-sectional groups. Group 1 was selected from an extensive histologic review and included 187 adult patients with NPs and 45 healthy control subjects. Group 2 was drawn from a prospective study and included 31 patients with NPs and 17 healthy control subjects. In this group nasal tissues were sampled after histologic examination to determine levels of inflammatory mediators, including MPO, IL-8, and IP-10. Group 3 was drawn from a prospective study of 48 patients with extensive polyps (score of 2 or 3 on both sides).

The primary efficacy end points were as follows: (1) change from baseline to end point values (on day 7) in the bilateral polyp size score and nasal resistance and (2) change from baseline in the nasal congestion score averaged over 7 days of treatment. The secondary end points included a change in TNSS or other individual nasal symptom score (loss of smell, anterior rhinorrhea, and postnasal drip score) averaged over 7 days of treatment from baseline values.

In group 3 a small portion of the polyp tissue was sampled by using endoscopic inspection to establish a baseline. Soon after sampling and histologic examination, the patients were evaluated for a therapeutic response to oral corticosteroids by administration of prednisone (30 mg once daily for 7 consecutive days). After that, polyp tissue was sampled again. Additionally, nasal secretions were collected to examine the levels of inflammatory mediators before and after treatment.

Immunohistochemical staining

Immunohistochemical staining was performed with the avidin-biotin-peroxidase method. Briefly, human nasal tissues embedded in paraffin were cut into 5- μ m sections and placed onto glass slides. After antigen retrieval, sections were stained with the murine mAbs anti-CD3 (1:100, Dako), anti-CD20 (1:200, Dako), anti-MBP (1:50, Dako), anti-HNE (1:50, Serotec), anti-IL-17 (1:100, R&D Systems), and anti-tryptase (1:200, Dako) and then detected by using streptavidin-biotin-horseradish peroxidase complex formation. The immunostaining was considered positive when brown cells were obtained after treatment with 3,3'-diaminobenzidine reagent. Sections stained by isotype-matched IgG instead of primary antibodies were used as negative controls. The sections were examined and scored by 2 independent observers who were blind to the diagnosis and clinical data. The numbers of positive cells were counted in 10 randomly selected hpfs ($\times 400$ magnification) and averaged.

The colocalization of eosinophils and neutrophils was examined with a MultiVision Polymer Detection System (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, after antigen retrieval, sections were stained with a primary antibody cocktail and then detected by using a MultiVision polymer cocktail (anti-rabbit/horseradish peroxidase plus

anti-mouse/alkaline phosphatase) and a solution of LVBlue and LVRed. As described previously, the sections were examined and scored by 2 independent observers who were blind to the diagnosis and clinical data. The numbers of positive cells were randomly counted in 10 hpfs ($\times 400$ magnification) and averaged.

Quantitative RT-PCR

Real-time RT-PCR was performed according to the manufacturer's instructions. Briefly, total RNA was extracted from nasal tissue with TRIzol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μ g of total RNA by using an oligo-(dT)18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. The expression of mRNA was determined by using an ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, Calif) and SYBR Premix Taq (TAKARA). The sequences of the primers (*CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10*) are listed in Table E1. The PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L of 5 μ mol/L primers, and 25 ng of synthesized cDNA in a 25- μ L reaction volume. Reactions were heated to 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 56°C to 60°C for 60 seconds. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. Expression of the target gene was expressed as the fold increase or decrease relative to the expression of β -actin. The mean value of the replicates for each sample was calculated and expressed as a cycle threshold (Ct). The level of gene expression was then calculated as the difference (Δ Ct) between the Ct value of the target gene and the Ct value of β -actin. The fold changes in the target gene mRNA levels were determined as $2^{-\Delta\Delta Ct}$.

ELISA

Freshly obtained tissue specimens were weighed, supplemented with PBS (1 mL of PBS/100 mg of tissues) and a protease inhibitor cocktail (Keygentec, Nanjing, Jiangsu, China), and homogenized on ice for 1 minute. The suspension was then centrifuged at 4000 rpm for 20 minutes at 4°C. The supernatants were stored at -80°C for ELISA. The cytokine concentrations were measured with commercially available ELISA kits (R&D Systems), according to the manufacturer's protocols. The detection limits were as follows: MPO, 1.56 mg/mL; IL-8, 3.9 pg/mL; and IP-10, 7.8 pg/mL. For convenient analysis, all values of less than the detectable limit were considered zero.

FlowCytomix analysis

Nasal secretions were collected as reported elsewhere. Briefly, open-cell flexible polyurethane foam was cut by a local distributor into sampler cubes of $30 \times 20 \times 10$ mm. The fluid-retention capacity of 1 sampler was 3 mL. The samplers were placed under direct visualization into each nasal cavity posterior to the mucocutaneous junction and left in place for 10 minutes. After removal of the samplers, fluid was extracted from each sampler by means of centrifugation. The protein levels of IFN- γ , IL-4, IL-5, IL-8, and IL-17 were quantified in nasal secretions by using a FlowCytomix kit (eBioscience). The detection limits for IFN- γ , IL-4, IL-5, IL-8, and IL-17 were 1.6, 3.0, 1.0, 0.5, and 2.5 pg/mL, respectively.

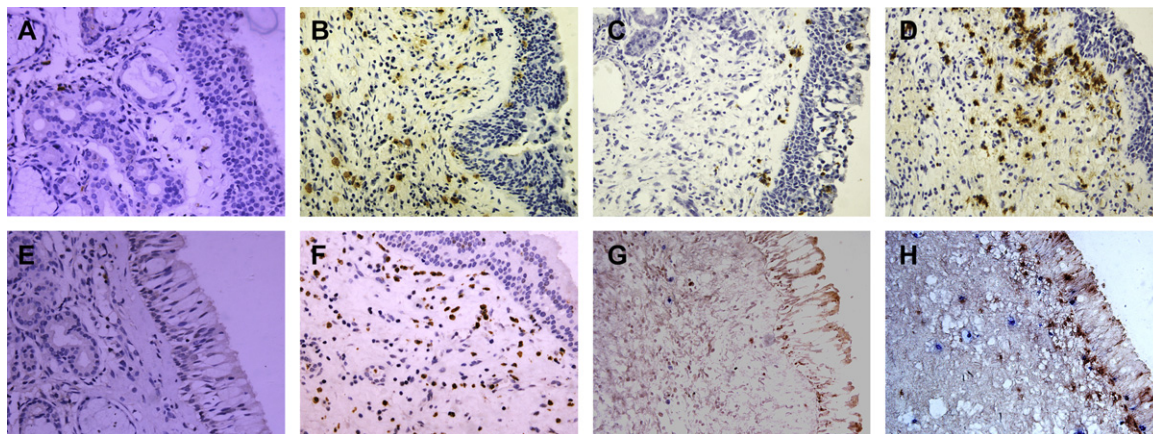


FIG E1. Immunohistochemical locations of eosinophils and neutrophils in NP and healthy control tissues. **A-F**, Representative staining of MBP⁺ cells (representing eosinophils), HNE⁺ cells (representing neutrophils), and IL-17⁺ cells is shown in healthy control (Fig E1, *A*, *C*, and *E*) and polyp tissues (Fig E1, *B*, *D*, and *F*) tissues. **G** and **H**, Representative double-staining of MBP⁺ cells (*blue*) and HNE⁺ cells (*brown*) is also shown in healthy control (Fig E1, *G*) and polyp (Fig E1, *H*) tissues.

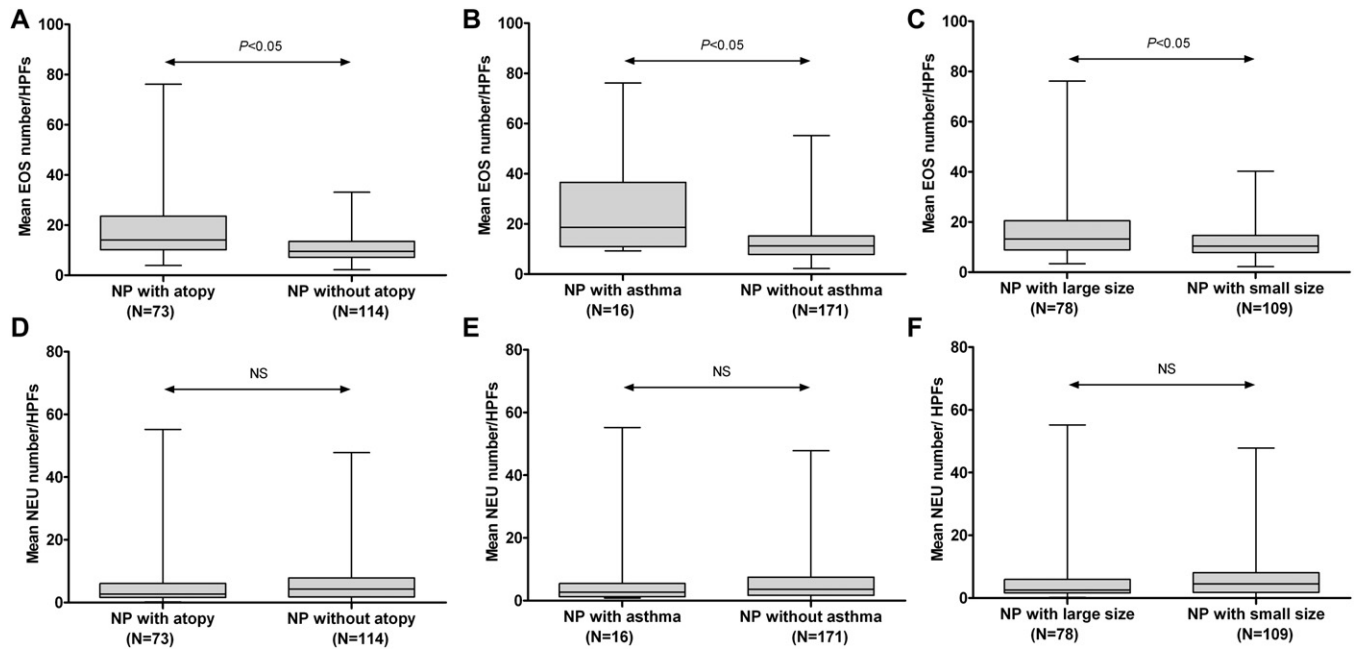


FIG E2. Numbers of eosinophils (EOS) and neutrophils (NEU) in patients with NPs with different polyp size, atopy, and comorbid asthma. **A-C**, Significantly higher levels of eosinophils were observed in patients with NPs with larger polyp size (Fig E2, A), atopy (Fig E2, B), and comorbid asthma (Fig E2, C). **D-F**, No significant increase in neutrophil numbers was observed in patients with larger polyp size (Fig E2, D), atopy (Fig E2, E), or comorbid asthma (Fig E2, F). Data are represented by a box-and-whisker plot, and Mann-Whitney U test was used for statistical analysis. *NP with large size*, NP size score is 3 on either 2 or 2 sides; *NP with small size*, NP size score is less than 3 on both sides; *NS*, not significant.

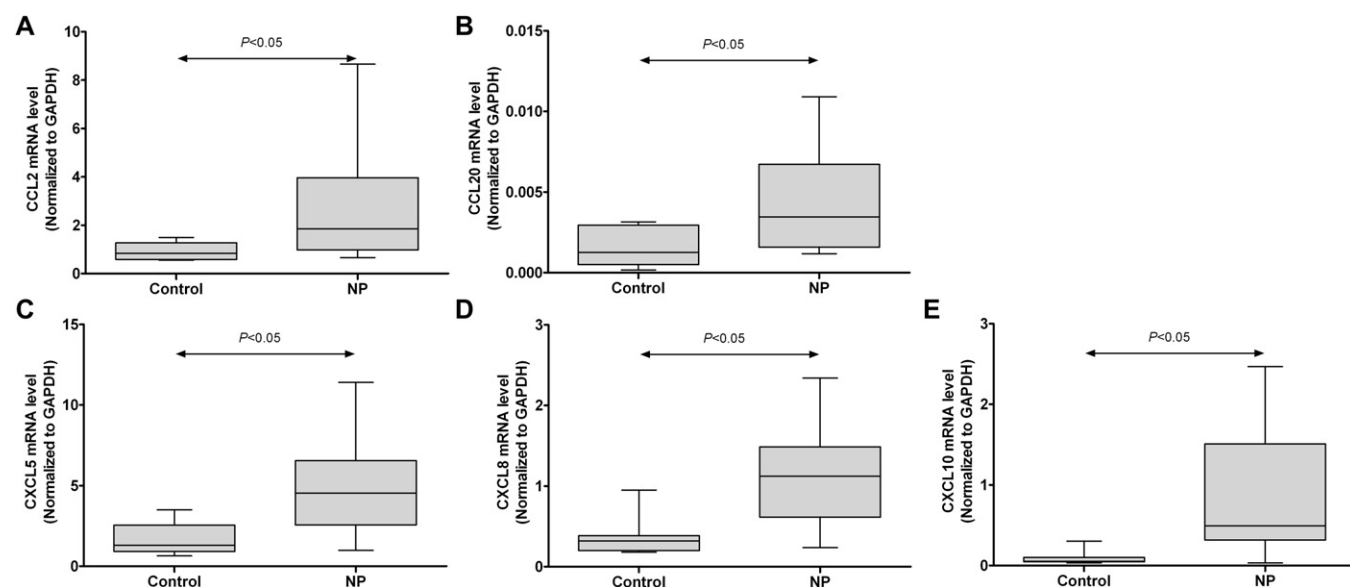


FIG E3. mRNA expression of chemokines (*CCL2*, *CCL20*, *CXCL5*, *CXCL8*, and *CXCL10*) in patients with NPs and healthy control subjects. mRNA levels of *CCL2* (A), *CCL20* (B), *CXCL5* (C), *CXCL8* (D), and *CXCL10* (E) were significantly increased in polyp tissues compared with those seen in healthy control tissues. Data are represented by a scatter plot. Solid bars represent median values, and the Mann-Whitney *U* test was used for statistical analysis.

TABLE E1. Primer sequences used for quantitative RT-PCR amplifications

	Forward 5'-3'	Reverse 5'-3'
<i>CCL2</i>	GCT GTG ATC TTC AAG ACC ATT GTG	TGG AAT CCT GAA CCC ACT TCT G
<i>CCL20</i>	GCG AAT CAG AAG CAG CAA GC	CTT CAT TGG CCA GCT GCC
<i>CXCL5</i>	GCT GTG TTG AGA GAG CTG CG	GGG CCT ATG GCG AAC ACT T
<i>CXCL8</i>	CAG TGC ATA AAG ACA TAC TCC AAA CC	GCA GTG TGG TCC ACT CTC AAT C
<i>CXCL10</i>	AAG CCA ATT TTG TCC ACG TGT T	TGGCCTTCGATTCTGGATTTC
β -actin	AAG ATG ACC CAG ATC ATG TTT GAG ACC	AGC CAG GTC CAG ACG CAG GAT