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29 Capsule Summary:

30 This study demonstrates nasal polyp tissue IL-25 level can be used for evaluating
31 clinical efficacy and serve as a predictor of oral-corticosteroid sensitivity in CRSwNP,
32 which contributes to the classifications and treatment of CRSwNP.
33 (34/35 words)

To the Editor:

Oral corticosteroids are commonly used in the treatment of chronic rhinosinusitis with nasal polyps (CRSwNP)¹. However, the efficacy of oral corticosteroid therapy varies between different CRSwNP patients. IL-25 mediated Th2-biased response is reported to be associated with corticosteroid sensitivity in airway inflammatory response², while their value for predicting oral-corticosteroid responds to CRSwNP has not been completely evaluated.

IL-25, an important epithelial-derived pro-inflammatory cytokine, plays a variety of roles in different inflammatory murine models, such as asthma, atopic dermatitis, and pulmonary fibrosis³, and orchestrates type 2 responses at the mucosa site. The correlation of IL-25 mediated Th2-biased response and corticosteroid sensitivity is expected to be involved in airway inflammatory response. Recently, our group has identified that local IL-25 plays a crucial role in promoting Th2-biased inflammation in CRSwNP, and may serve as a promising therapeutic target in CRSwNP^{E1}. Moreover, we have demonstrated that nasal tissue IL-25 level is a novel biomarker for CRSwNP patients with airway hypersensitiveness^{E2}. Cheng et al. reported that the IL-25^{high} asthmatic patients have better improvements in FEV1 following inhaled corticosteroid treatment than in the IL-25^{low} subgroup², and suggested serum IL-25 levels as a predictor for corticosteroid sensitivity in asthma therapy. This study thus aims to characterize the inflammatory profiles of CRSwNP patients, and to evaluate the predictive biomarkers for oral-corticosteroid responses in a prospective clinical study. The study was approved by the Ethical Committees of the First and Fifth Affiliated Hospitals of Sun Yat-sen University (clinical-trial registration no. ChiCTR-BON-16010179 and NCT02110654). An informed consent was obtained from each subject.

We enrolled 52 patients with CRSwNP in this study, who underwent a 2-week oral prednisone treatment and divided into 2 subgroups (**Fig. 1A** and **Table E1**, corticosteroid sensitive and non-sensitive subgroups)^{E3}. Our results showed that there were 29 corticosteroid sensitive CRSwNP patients and 23 CRSwNP non-sensitive

patients. Clinical characteristics are listed in **Table E2**.

Nasal polyps (NP) specimens and blood samples were obtained before oral-corticosteroid treatment. The inflammatory profiles were evaluated by means of Hematoxylin-eosin (HE) and immunohistochemistry (IHC) staining, ELISA, and luminex-based multiplex assay. Data were expressed as medians and interquartile ranges (IQRs). The predictive ability of the parameters was evaluated by receiver operating characteristic (ROC) curve. A *P* value less than 0.05 was considered as statistically significant (details in online supplement).

Our results showed that the protein levels of IL-25 in NP tissues and in serum, as well as the NP endoscopic scores were significantly elevated in corticosteroid sensitive subgroup compared to the non-sensitive subgroup (**Fig. 1B-C** and **Table E2**). The immunoreactivity of IL-25 was significantly increased in the corticosteroid sensitive subgroup (**Fig. 1D-E**). HE and IHC staining showed eosinophil, but not neutrophil counts were significantly increased in corticosteroid sensitive subgroup (**Fig. E1. A-D**). Similarly, a significantly increased eosinophil, but not neutrophil counts were found in peripheral blood of the corticosteroid sensitive subgroup (**Fig. E1. E-F**). Using tissue luminex-based multiplex assay and serum ELISA, we also found that serum IL-5 level was significantly increased in corticosteroid sensitive subgroup compared to the non-sensitive subgroup (**Fig. E2**).

Next we further performed logistic regression analysis to determine the factors associated with corticosteroid efficacy by between-group comparison analysis followed by ROC curve analysis. The following variables were introduced into the multivariate model: the endoscopy scores, tissue IL-25 levels and eosinophil counts, serum IL-5, IL-25 levels and peripheral blood eosinophil counts. The multivariate analysis showed that corticosteroid sensitivity was significantly associated with tissue and serum IL-25 levels (both $P < 0.05$), and marginally associated with tissue eosinophil counts ($P = 0.05$, **Table E3**) in this cohort. Sensitivity analysis by including individuals with robust NPs (NP endoscopy scores ≥ 3) also showed similar results (**Table E4-6**).

Following these observations, we further examined the sensitivity and specificity of

NP tissue and serum IL-25 levels in predicting oral-corticosteroid sensitivity in this CRSwNP cohort using an ROC curve analysis. As shown in **Fig. 2A**, the ROC curve analysis indicated that the NP tissue IL-25 levels (area under the curve (AUC) = 0.963) had a higher predictive value for oral-corticosteroid sensitivity than those of the serum IL-25 levels. The best cutoff point for NP tissue IL-25 level was determined using the Youden index (based on the optimal sensitivity and specificity), in order to discriminate oral-corticosteroid sensitivity and non-sensitivity in CRSwNP patients. Our data demonstrated that a cutoff point of 22.50 pg/mL NP tissue IL-25 level provided a Youden index of 81.5%; with a sensitivity of 85.7% and a specificity of 95.8%; and the positive and negative predictive values of 96% and 85.2% for oral-corticosteroid sensitivity (**Fig. 2B**).

While oral-corticosteroids are one of the frequently used treatments in the management of CRSwNP¹; there is lack of effective means of predicting corticosteroid sensitivity in CRSwNP patients. It was reported that the overall sensitivity rates of topical corticosteroid therapy to NP were only between 53.8-80%^{1, 4, 5}. Hissaria et al.⁶ reported that 36% of CRSwNP patients were non-sensitive to corticosteroid therapy based on the thirty-one-item rhinosinusitis outcome measure scores. Milara et al.⁷ found that about 27% of CRSwNP patients with a high expression of Mucin 4 were resistant to 15-day oral-corticosteroid therapy, inferring that the high expression of Mucin 4 participated in corticosteroid resistance. Our results also showed that 44.2% (23/52) of CRSwNP patients were non-sensitive to 14-day oral-corticosteroid therapy based on the nasal endoscopic scores^{E3}. A recent retrospective cohort study including more than 200,000 asthmatic patients⁸ revealed that exposure to 4 or more prescriptions of oral-corticosteroid therapy within a year was associated with significantly greater odds of adverse effects (AEs) for osteoporosis, fractures, gastrointestinal ulcers/bleeds, cataracts, obesity, type 2 diabetes, and hypertension (odds, 1.21-1.44 depending on the AEs). Each prescription might result in a cumulative burden on current and future health regardless of dose and duration, which signify the need for careful and accurate prescription of corticosteroid in CRSwNP management.

It is thus important to develop useful methods for identifying distinct endotypes of CRSwNP and predicting responses to oral-corticosteroid therapy. To this aim, we previously reported that the NP neutrophilia impaired oral-corticosteroid sensitivity of CRSwNP patients⁹, but the key factor determining oral-corticosteroid sensitivity remains elusive. In this study, we further identified the correlation of oral-corticosteroid sensitivity and predictive biomarkers in CRSwNP oral-corticosteroid therapy. All data indicated that the NP tissue IL-25 level; and to a certain extent, serum IL-25 can be used for predicting clinical efficacy of oral-corticosteroid sensitivity in CRSwNP patients, which might expand our understanding on CRSwNP endotypes and contribute to the basis of personalized treatment for CRSwNP. Additionally, the use of NP tissue IL-25 level as a biomarker may also enhance the quality of life of non-sensitive patients whereby it shortens the time frame to receive the appropriate treatment by at least 14 days while preventing unnecessary steroid usage and side effects mentioned.

Key words

Chronic rhinosinusitis; corticosteroid sensitivity; interleukin 25; nasal polyps

Abbreviations

AE, adverse effect; AUC, area under the curve; CRS, chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin-eosin; IHC, immunohistochemistry; IQRs, interquartile ranges; NP, nasal polyps; OR, odds ratio; ROC, receiver operating characteristic.

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Competing interests

The authors have declared that no competing interests exist.

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Figure legends

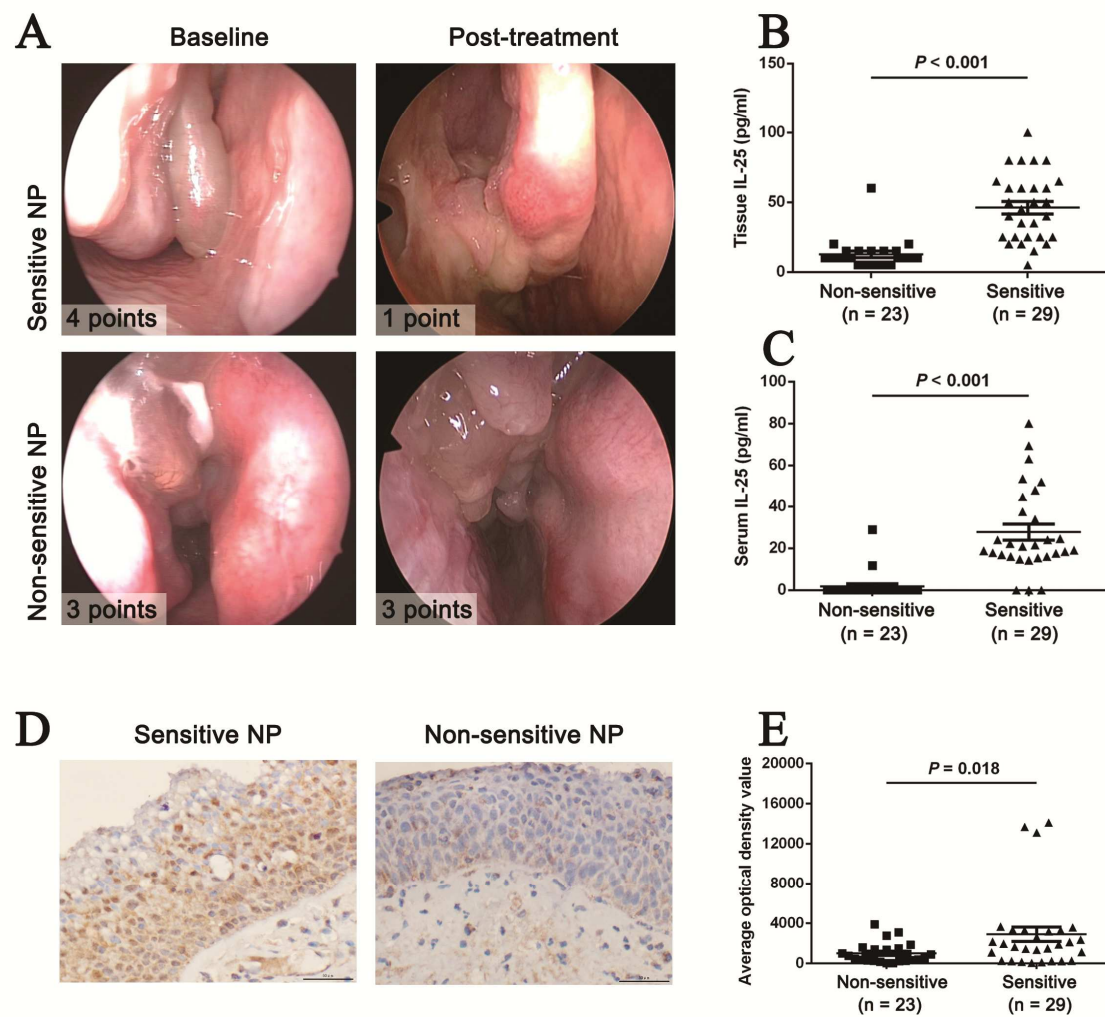
Fig 1. Increased expression of IL-25 in the corticosteroid sensitive subgroup of CRSwNP

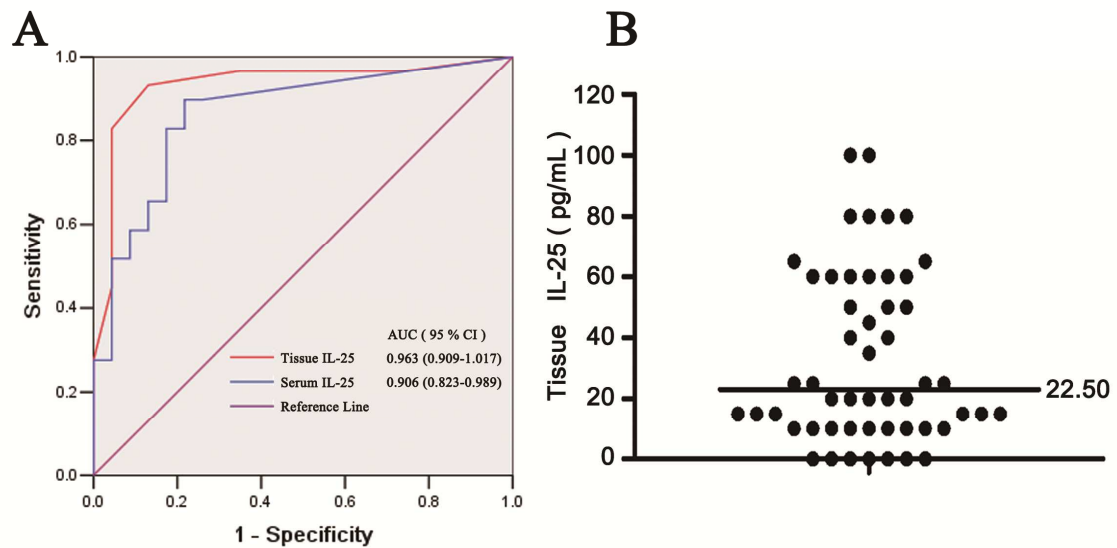
(A) Representative nasal endoscopic images of baseline and post-treatment with oral prednisone in corticosteroid sensitive and non-sensitive subgroups; The concentrations of tissue (B) and serum

(C) IL-25 in the two subgroups; The immunoreactivity of IL-25 (magnification, 400×) (D-E) in the two subgroups by means of immunohistochemistry.

Fig 2. Receiver operating characteristic (ROC) curve and optimal cutoff points for corticosteroid sensitivity

(A) The ROC curve of nasal polyps tissue and serum IL-25 levels for corticosteroid sensitivity. Optimal cutoff points of tissue IL-25 level (B) for corticosteroid sensitivity.





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Materials and methods

This study was approved by the Ethical Committees of the First and Fifth Affiliated Hospitals of Sun Yat-sen University (clinical trial registration no. ChiCTR-BON-16010179 and NCT02110654). An informed consent was obtained from each subject.

Patients and tissue samples

Fifty-two CRSwNP patients were recruited from the First and Fifth Affiliated Hospital of Sun Yat-sen University (Guangzhou and Zhuhai, China). The diagnosis of CRSwNP was carried out according to the European position paper on rhinosinusitis and nasal polyps (EPOS 2012)^{E4}. The total nasal symptom score (TNSS) was calculated as the sum of scores for 4 individual symptoms at the same time^{E4, 5}. The CRSwNP patients, with TNSS [VAS] over 5, were enrolled in this prospective study. First of all, they were prescribed with oral corticosteroids (30 mg of prednisone once daily for 14 days). Before the treatment, polyp tissues were sampled from each subject, as well as TNSS recorded. Biopsies of two small pieces of nasal polyp tissue, about 2×2 mm in each size (25 mg), were taken from each patient involved just under the horizontal portion of middle turbinate. One is for Hematoxylin-eosin (HE) and immunohistochemistry (IHC) staining, and the other is for luminex-based multiplex assay. Non-sensitive or resistant CRSwNP subgroup was defined as described by Milara et al.^{E6}, namely patients who are unable to reduce more than 1 point by the nasal polyp scoring system after oral corticosteroid management (**Fig. 1** and **Table E3**). All nasal endoscopic images were evaluated and scored by 2 otolaryngologists independently who were blinded to the design of the trial. The atopic status was evaluated by a skin prick test and the serum IgE (Phadia, Uppsala, Sweden) specific to common inhalant allergens (e.g. pollens, house dust mites, pets, molds, and cockroaches). None of the patients included used oral or nasal steroids or other immunomodulatory drugs within 4 weeks prior to surgery. Blood samples were also collected before the treatment, and the peripheral blood eosinophil counts were performed using an automated analyzer.

HE and IHC staining

NP tissues embedded in paraffin were cut into 5- μ m sections and placed onto glass slides. HE and IHC staining were performed for histological examination using the standardized procedures as previously described^{E7}. The primary antibodies, including rabbit anti-IL-25 antibody (Abcam, Cambridge, MA, 1:250) and rabbit anti-MPO antibody (Abcam, 1:200), were used to identify IL-25⁺ cells and neutrophils. Negative control studies were performed by replacing the primary antibodies with normal IgG in appropriate concentrations.

The slides were blindly examined and coded with no awareness of the clinical data. The immunoreactivity of five randomly selected subepithelial areas (400 $\times$) on each slide of the subjects was quantitatively scored by using Image-Pro Plus 6.0 software, and the average optical density (AOD) value and the counts of positive cells were calculated.

ELISA

The levels of IL-4, IL-5, IL-13, IFN- γ , IL-17A and IL-25 in blood samples were measured by ELISA (R&D Systems) according to the manufacturer's instructions, and as previously described^{E7}.

Luminex-based multiplex assay

Protein extracted from polyp tissues was measured by luminex-based multiplex assay according to the manufacturer's protocol^{E8}. Total protein concentration in the supernatants that determined by using a standard BCA assay was normalized to a concentration of 50 μ g total protein/20 μ L per sample. All samples were analyzed for IL-25, IFN- γ , IL-12, IL-1 β , IL-8, IL-4, IL-5, IL-13, EOTAXIN, EOTAXIN-2, EOTAXIN-3, TNF- α , IL-17A, IL-23, IL-6, MCP-1, MCP-4, IL-9, and IL-10 using Luminex-based Multiplex kits (Millipore).

Statistical analysis

Data were expressed as the medians and interquartile ranges (IQRs) and analyzed

using the Kruskal-Wallis H test and nonparametric Mann-Whitney U test unless otherwise stated. Logistic regression analysis was performed to show the significantly difference of clinical parameters between corticosteroid-sensitive and non-sensitive NP groups and the odds ratios and 95% confidence intervals of the clinical parameters were also calculated in the logistic regression model. The predictive ability of each parameter was evaluated by receiver operating characteristic (ROC) curve. Nonparametric Z tests (2-tailed) were used for ROC curve area comparisons. A P value less than 0.05 was considered as statistically significant.

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Fig E1. The distinct histological characteristics in corticosteroid sensitive and non-sensitive subgroups

(A) Representative HE-stained histological sections in the two subgroups (magnification, 400×); (C) The immunoreactivity of MPO in the two subgroups (magnification, 400×); (B and D) The counts of tissue eosinophils and neutrophils in the two subgroups; (E and F) The counts of peripheral blood eosinophils and neutrophils in the two subgroups. Data are expressed as median (IQRs).

Fig E2. The distinct protein expression profiles in corticosteroid sensitive and non-sensitive subgroups

(A) Shown is the heatmap of cytokines upregulated or downregulated in NP tissues of the two subgroups, as determined by Luminex-based multiplex assay. (B-F) The protein levels of serum cytokines, IL-4, IL-5, IL-13, IFN- γ , and IL-17A, in the two subgroups. Data are expressed as median (IQRs).

Table E1. Polyp scoring system

Table E2. Clinical characteristics of CRSwNP patients in corticosteroid sensitive and non-sensitive subgroups

Table E3. ORs for 2 molecular markers in CRSwNP patients

Table E4. Clinical characteristics of CRSwNP patients, whose nasal polyp scores were more than 3 points, in corticosteroid sensitive and non-sensitive subgroups

Table E5. Biologic markers of CRSwNP patients, whose nasal polyp scores were more than 3 points, in corticosteroid sensitive and non-sensitive subgroups

Table E6. ORs for 2 molecular markers in CRSwNP patients, whose nasal polyp scores were more than 3 points

Table E1. Polyp scoring system (illustration was drawn based on the study reported by Milara et al. ^{E8}).

Polyp score	Polyp size	
0	No polyps	
1	Small polyps in the middle meatus not reaching below the inferior border of the middle concha.	
2	Polyps reaching below the lower border of the middle turbinate.	
3	Large polyps reaching the lower border of attachment of the inferior turbinate or polyps medial to the middle concha.	
4	Large polyps causing almost complete congestion/obstruction of the inferior meatus.	

Table E2. Clinical characteristics of CRSwNP patients in corticosteroid sensitive and non-sensitive subgroups

	Sensitive (n=29)	Non-sensitive (n=23)	P Value
Age, mean(SD), (y)	33.6±11.1	37.8±8.34	0.14
Sex (male/female)	17/12	14/9	0.90
Atopy (yes/no)	16/13	11/12	0.81
Symptom scores mean (SD)	16.93±5.96	17.43±6.19	0.77
NP endoscopic scores mean (SD)	4.21±1.47	3.39±1.34	0.04
CT scores, mean (SD)	12.52±7.44	9.43±4.47	0.09
Total IgE, mean (SD), (IU/mL)	72.12±29.22	72.34±30.36	0.98

Definition of abbreviations: CT=computed tomography; NP=nasal polyps.

Table E3. ORs for 3 molecular markers in CRSwNP patients

Variables	OR	95% CI	P Value
Tissue IL-25	1.167	1.061-1.285	< 0.01
Serum IL-25	1.176	1.037-1.335	0.01
Tissue eosinophilis	1.149	0.972-1.359	0.05

Definition of abbreviations: CI=confidence interval; OR= odds ratio.

Table E4. Clinical characteristics of CRSwNP patients , whose nasal polyp scores were more than 3 points, in corticosteroid sensitive and non-sensitive subgroups

	Sensitive (n = 24)	Non-sensitive (n = 17)	P Value
Age, mean(SD), (y)	35.0±11.2	38.2±7.98	0.32
Sex (male/female)	14/10	10/7	0.98
Atopy (yes/no)	12/12	9/8	0.85
Symptom scores mean (SD)	17.42±6.21	17.12±6.03	0.88
NP endoscopic scores mean (SD)	4.67±1.17	4±1.06	0.07
CT scores, mean (SD)	12.92±7.37	9.76±4.74	0.13
Total IgE, mean (SD), (IU/mL)	72.44±28.72	74.08±34.28	0.87

Definition of abbreviations: CT=computed tomography, NP=nasal polyps.

Table E5. Biologic markers of CRSwNP patients, whose nasal polyp scores were more than 3 points, in corticosteroid sensitive and non-sensitive subgroups

	Sensitive (n = 24)	Non-sensitive (n = 17)	P Value
Tissue (pg/mL)			
IL-25	47.08±25.91	14.41±12.61	< 0.01
IFN- γ	16.95±24.58	26.75±24.98	0.22
IL-12	8.68±3.72	8.48±2.48	0.85
IL-1 β	6.23±2.05	5.71±1.38	0.37
IL-8	468.22±241.13	336.37±280.27	0.11
IL-4	89.24±62.87	88.91±51.77	0.99
IL-5	2.18±1.72	2.77±3.33	0.46
IL-13	16.94±8.18	15.93±6.22	0.67
EOTAXIN	55.29±40.42	67.40±64.35	0.46
EOTAXIN-2	193.44±160.96	217.97±144.15	0.62
EOTAXIN-3	544.97±686.14	613.93±591.24	0.74
TNF- α	7.27±2.04	6.27±1.76	0.11
IL-17A	5.10±2.59	5.73±2.38	0.43
IL-23	76.69±9.01	76.98±8.02	0.92
IL-6	19.93±10.41	28.50±18.93	0.07
MCP-1	641.56±287.53	588.45±354.99	0.60
MCP-4	36.65±9.48	40.99±12.43	0.21
IL-9	9.37±4.00	10.23±4.32	0.52
IL-10	931.28±110.26	1076.51±1252.39	0.57
Tissue EOS counts (HPF × 400)	34.39±16.95	20.28±9.18	< 0.01
Peripheral blood EOS counts (10⁹/L)	0.32±0.17	0.26±0.16	0.26
Serum (pg/mL)			
IL-25	26.38±19.61	2.39±7.41	< 0.01
IFN- γ	29.92±27.33	30.67±14.05	0.92
IL-4	ND	ND	--
IL-5	37.67±13.32	24.72±9.02	< 0.01
IL-13	39.01±70.0	29.62±44.40	0.63
IL-17A	6.53±19.09	9.98±18.08	0.56

Definition of abbreviations: EOS = eosinophil , HPF = high power field; IFN- γ = interferon gamma; MCP = monocyte chemoattractant protein; ND=not detectable; TNF- α = tumor necrosis factor- α .

Table E6. ORs for 2 molecular markers in CRSwNP patients, whose nasal polyp scores were more than 3 points

Variables	OR	95% CI	P Value
Tissue IL-25	1.193	1.079-1.318	< 0.01
Serum IL-25	1.145	1.033-1.269	0.01

Definition of abbreviations: CI=confidence interval; OR= odds ratio.

