Neurohormonal markers in chronic rhinosinusitis

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Chronic rhinosinusitis (CRS), especially with nasal polyps, continues to elude precise pathogenesis and effective treatment. Prior work in our laboratory demonstrated interleukin-33 (IL-33) and Substance P (SP) activation of mast cells, and inhibitory effect of interleukin-37 (IL-37). Our objective is to study the expression of these neurohormonal mediators in mast cell stimulation of nasal polyposis. This was a prospective research study involving collection of nasal lavage fluid and nasal polyp tissue from adult patients with CRS. The study was divided into two arms. First, nasal lavage fluid was collected from normal controls, and patients with allergic rhinitis, CRS, or CRS with nasal polyposis. The second arm was collection of nasal tissue from normal controls undergoing inferior turbinoplasty, or patients with nasal polyposis. Enzyme-linked immunosorbent assay and quantitative polymerase chain reaction techniques were used to determine levels in the lavage fluid and relative gene expression in the tissue of SP, IL-33, and IL-37. In total, 70 lavage and 23 tissue specimens were obtained. The level of SP was highest in patients with polyps; however, gene expression was reduced compared to normal controls. The level of IL-33 was reduced in patients with polyps as compared to patients with allergy and sinusitis, and its gene expression was not significantly different from normal controls. IL-37 was elevated in the lavage fluid of patients with nasal polyps and its gene expression was increased in the polyp tissue. Levels of SP and IL-37 were elevated in the lavage fluid of patients with nasal polyps as compared to normal controls and other sinonasal pathologies, and gene expression of IL-37 was significantly increased in the polyp tissue itself. These findings implicate these neurohormonal molecules in the pathophysiology of nasal polyposis and provide possible novel therapeutic targets.

Nasal polyps are benign inflammatory masses that arise from the nasal mucosa, representing a subgroup of chronic rhinosinusitis (CRS) (1). The pathophysiology of CRS with nasal polyposis (CRSwNP) is complex, involving both host and environmental factors. Proposed mechanisms include

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defects in the airway epithelial barrier, microbial surface colonization, activation of the body's innate and adaptive immune system, and tissue remodeling (2). The mast cell (MC), the body's major effector cell of allergic reactions, is found in high numbers in nasal polyps (3). MC are stimulated via exposure to an antigen that cross-links a specific immunoglobulin, but can also be triggered by cytokines, neuropeptides, and microbes (4). While MC are associated with nasal polyposis, the precise mechanism of this process has not been elucidated (3-7).

Our laboratory has shown that SP, a pro-inflammatory peptide secreted by sensory neurons, can activate MC (8). SP has a widespread distribution in the central and peripheral nervous systems with effects on nearly all organ systems (9). The role of SP in other inflammatory disease processes, such as arthritis, has been studied extensively (10, 11), but there have been few reports describing its role in nasal polyposis (12-14). In a pilot study, we demonstrated that SP was detectable in the nasal lavage fluid of patients with CRSwNP (15). Previous work from our laboratory also reported that the action of SP on vascular endothelial growth factor (VEGF) in MC was enhanced by the "alarmin" cytokine IL-33 (16). The expression of IL-33 was reported to be elevated in nasal polyposis in association with eosinophilia (17), and this is mirrored in other eosinophilic-mediated conditions such as eosinophilic esophagitis and asthma (18-19). In contrast, however, one recent study demonstrated significantly reduced tissue levels of IL-33 in those patients with CRSwNP *versus* those without nasal polyps (CRSsNP) (20). The expected pattern of IL-33 release in nasal polyposis and its relationship to SP-activation of MC is thus not fully defined.

One additional cytokine that warrants study in the mechanism of nasal polyposis is IL-37, a unique anti-inflammatory molecule that binds to the IL-18-receptor and inhibits release of pro-inflammatory cytokines (21). In experimental studies, IL-37 protected mice against inflammation including lung and spinal cord injury, coronary artery disease, and arthritis (22). Mucosal MC-stimulated cellular inflammatory response in the gut could be inhibited by IL-37 (23). IL-37 has been infrequently studied in nasal polyposis. One study showed that IL-37 levels in nasal secretions were reduced in concert with increased eosinophilic inflammation in patients with eosinophilic CRSwNP (24). A similar study reported significantly decreased mRNA and protein expression of IL-37 in nasal polyp tissue (25).

The aim of this study was to investigate levels and gene expression of SP, IL-33, and IL-37 in patients with CRSwNP, CRSsNP, allergic rhinitis, and normal controls.

MATERIALS AND METHODS

Participants

Patients were evaluated in the otolaryngology clinic at Tufts Medical Center (TMC) as part of their clinical care between July 2017 and March 2019. Consent was obtained from all patients participating in the study. Patients were recruited for collection of nasal lavage and collection of nasal tissue. Approval for this study was obtained from the TMC Institutional Review Board.

Nasal lavage

Nasal lavage samples were collected from four groups: (i) normal controls, (ii) allergic rhinitis, (iii) CRSsNP, and (iv) CRSwNP. The controls consisted of patients requiring nasal endoscopy for an unrelated complaint such as epistaxis or patients who were found to have negative allergy testing and no evidence of sinusitis or polyposis. The lavage technique consisted of nasal endoscopy performed with a rigid endoscope to facilitate direct suctioning of 0.5 mL of mucous into a Lukens trap® (Cardinal Health, OH, USA) from along the nasal floor using a rigid suction. The samples were mixed with 1 mL of sterile normal saline to clear any mucous stuck in the trap. The samples were subsequently centrifuged at 350 g for 10 minutes; the supernatant fluid was collected, labeled, and stored at -80°F until further analysis.

Nasal tissue

The second arm of the study consisted of collection of nasal tissue from two groups: (i) normal controls, and (ii) patients with CRSwNP. In both groups, specimens were collected in the operating room as part of the surgical-procedure. Polyps were sampled with a forceps and brought to the laboratory on ice. They were then

cut into two specimens, one of which was treated with RNAlater (Thermo Fisher Scientific, MA); both were stored at -80°F until further analysis. Control tissue in this setting consisted of a small sample of mucosa taken from the anterior face of the inferior turbinate in patients undergoing inferior turbinate reduction. Such tissue was processed similarly to the polyp tissue.

Mediator levels in lavage fluid

To determine the levels of mediators in the lavage fluid, enzyme-linked immunosorbent assay (ELISA) was employed. Specifically, ELISA kits were used to measure lavage SP (Phoenix Pharmaceuticals, Inc., CA, USA), IL-33 and IL-37 (R&D Systems, MN, USA) according to manufacturer's protocols.

Determination of relative gene expression

To determine relative gene expression, 30 mg of specimen that had been stored in RNAlater (Thermo Fisher Scientific, MA, USA) was weighed. Total RNA was then isolated using the RNeasy Mini Kit (Qiagen, CA, USA) per manufacturer's protocol. Reverse transcription was performed with 200 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was then performed using TaqMan gene expression assays with validated oligonucleotide primers (Applied Biosystems, CA, USA). Samples were run for 45 cycles using the Applied Biosystems 7300 Real-Time PCR system. Relative mRNA abundance was determined from standard curves run for each experiment. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase endogenous control.

Statistical analysis

All data were validated and inspected for outliers. The results are presented as scattergrams, with symbols representing individual data points and the horizontal lines representing the median and interquartile range for each group. Normality of distribution was checked with the Shapiro-Wilk test. Comparisons between the nasal polyp group and control groups were performed using the nonparametric Mann-Whitney U test. A result was considered significant at a p-value of <0.05. The analysis was performed by using the GraphPad Prism version 8.0 software (GraphPad Software, CA, USA).

RESULTS

Demographics

In total, 70 nasal lavage samples were collected from controls and patients with allergic rhinitis, CRSsNP, and CRSwNP (Table I). The allergic rhinitis group was younger (median age 35 years) and had a greater female representation (84%) than the other groups of patients. Asian ethnicity was represented

Table I. *Demographic profile of groups collected for nasal lavage fluid*

	NC	AR	CRSsNP	CRSwNP
Number	15	19	13	23
Age (median)	56	35	51	56
Gender (N, %)				
Female	7 (47%)	16 (84%)	8 (62%)	8 (35%)
Male	8 (53%)	3 (16%)	5 (38%)	15 (65%)
Ethnicity (N, %)				
Caucasian	9 (60%)	6 (32%)	8 (62%)	12 (52%)
Black or African American	2 (13%)	0 (0%)	0 (0%)	2 (9%)
Asian	2 (13%)	12 (63%)	3 (23%)	7 (30%)
Latino	2 (13%)	1 (5%)	2 (15%)	2 (9%)

NC: normal controls; AR: allergic rhinitis; CRSwNP: chronic rhinosinusitis with nasal polyposis; CRSsNP: chronic rhinosinusitis without nasal polyposis.

to a greater extent in patients with allergic rhinitis (63%) and patients with CRSwNP (30%) than in other groups. For tissue sampling, 20 polyp samples were collected, and three normal control samples were collected from the anterior surface of the inferior turbinate (Table II). The groups were roughly similar in age; however, the normal control subset did not include any female subjects. Caucasian ethnicity was represented in 50% of patients, Asian ethnicity in 30% of patients, and Black or African American ethnicity in 20% of the patients from whom polyp tissue was collected.

Substance P

SP was detected in the nasal lavage fluid from the four study groups (Fig. 1). The median level was highest in the CRSwNP group (2.70 ng/mL), which was significant when compared to patients with allergic rhinitis (0.92 ng/mL, p = 0.001), but not when compared to CRSsNP (2.09 ng/mL) and

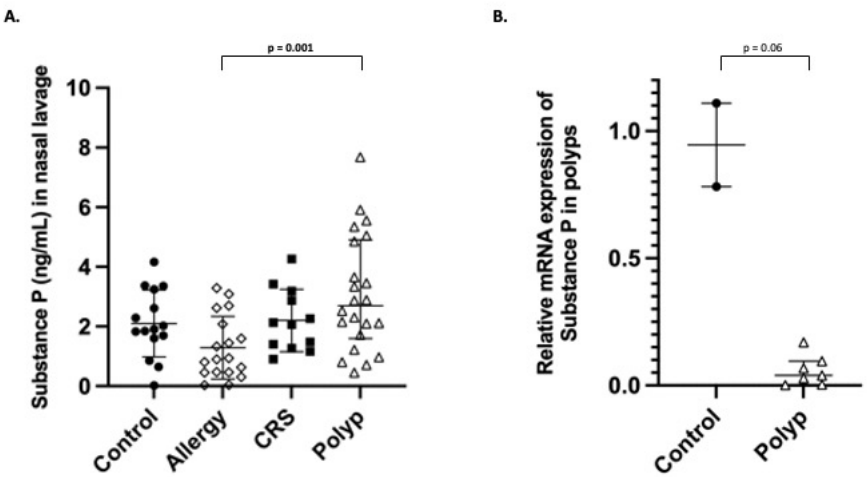

Fig. 1. ***A)*** *Concentrations of substance P in the nasal lavage fluid of patients with nasal polyps versus normal controls, allergy, and chronic rhinosinusitis (CRS).* ***B)*** *Relative mRNA expression in nasal polyp tissue as compared to normal nasal mucosa. Significant differences are denoted with a horizontal bar and p-value.*

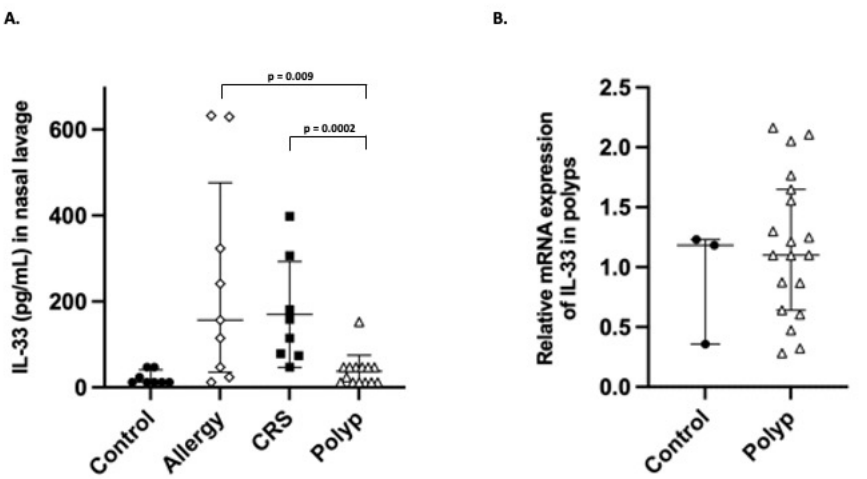

Fig. 2. ***A)*** *Concentrations of interleukin-33 in the nasal lavage fluid of patients with nasal polyps versus normal controls, allergy, and chronic rhinosinusitis (CRS).* ***B)*** *Relative mRNA expression in nasal polyp tissue as compared to normal nasal mucosa. Significant differences are denoted with a horizontal bar and p-value.*

normal controls (1.91 ng/mL). The gene expression of SP trended towards reduced in nasal polyp tissue as compared to normal controls (p = 0.06) (Fig. 1).

Interleukin-33

Median levels of IL-33 were significantly decreased in patients with CRSwNP (35.15 pg/mL) as compared to patients with allergic rhinitis (156.6 pg/mL, p=0.009) and patients with CRSsNP (158.2 pg/mL, p=0.0002) (Fig. 2). There was no statistical difference between levels in patients with CRSwNP as compared to controls (11.70 pg/mL). There was also no difference in relative mRNA expression between polyp tissue and normal nasal tissue (Fig. 2).

Interleukin-37

IL-37 was significantly increased in the lavage fluid of patients with CRSwNP (523.4 pg/mL) as compared to patients with CRSsNP (17.06 pg/mL, p=0.013), allergic rhinitis (20.11 pg/mL, p=0.001), and normal controls (32.42 pg/mL; p = 0.035) (Fig. 3). Relative expression of IL-37 mRNA was significantly increased in the polyp tissue as compared to normal nasal tissue (p= 0.038).

Table II. *Demographic profile of groups collected for tissue sampling*

	Control	Polyp
Number	3	20
Age (median)	53	56
Gender (N, %)		
Female	0 (0%)	6 (30%)
Male	3 (100%)	14 (60%)
Ethnicity (N, %)		
Caucasian	2 (67%)	10 (50%)
Black or African American	0 (0%)	4 (20%)
Asian	0 (0%)	6 (30%)
Latino	1 (33%)	0 (0%)

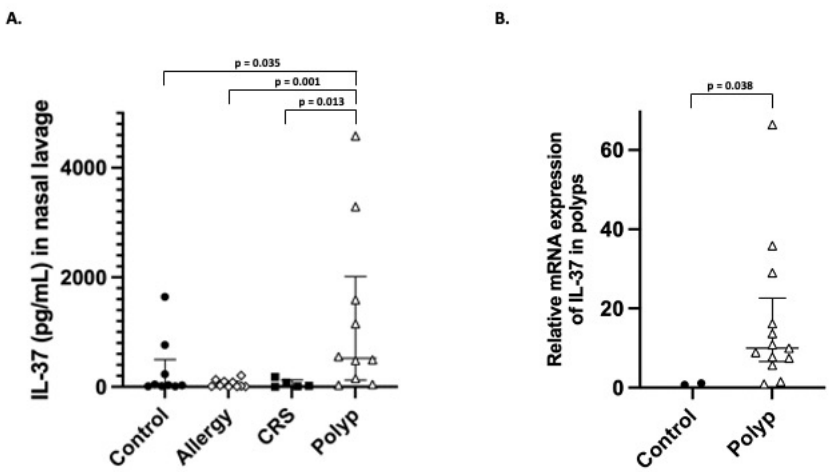

Fig. 3. ***A)*** *Concentrations of interleukin-37 in the nasal lavage fluid of patients with nasal polyps versus normal controls, allergy, and chronic rhinosinusitis (CRS).* ***B)*** *Relative mRNA expression in nasal polyp tissue as compared to normal nasal mucosa. Significant differences are denoted with a horizontal bar and p-value.*

DISCUSSION

In this study, the expression of neuroimmune mediators was studied in nasal lavage fluid and nasal tissue in nasal polyposis. Overall, we found different patterns among the three neuroimmune mediators studied: SP, IL-33, and IL-37.

Other studies have demonstrated SP-positivity in tissue biopsies of nasal polyps (12) and increased levels of SP in nasal polyps relative to serum levels (13). In 1995, Baumgarten et al. studied SP in the nasal mucosa of patients with and without allergies, noting that SP enhances mediator response to allergen (14). Here, we show that SP levels tend to be higher in the nasal lavage fluid of patients with nasal polyps as compared to other types of sinonasal pathology, in particular allergy patients. On the other hand, the gene expression of SP mRNA in the polyp tissue itself is decreased. These findings are at odds with previous findings of elevated SP levels in allergic rhinitis and nasal polyposis, though few reports have studied levels of SP across different disease processes as done in the present study (12-14). In terms of pathogenesis of nasal polyps, these results may suggest one of two possibilities. First, SP may be released into the lavage fluid from the nasal polyp tissue, and therefore is more easily detected in lavage fluid than in the tissue itself. Second, it is possible that nasal polyps are not well-innervated with sensory neurons that produce SP; this molecule might diffuse from other parts of the nasal mucosa.

We show that IL-33 is highest in the nasal lavage fluid of patients with CRSsNP and allergic rhinitis, but not in patients with CRSwNP. Similarly, we did not detect a difference in gene expression of IL-33 in the polyp tissue. These findings suggest that IL-33 is downregulated in the process of nasal polyposis. IL-33 is released primarily from endothelial and epithelial cells. Polyps mostly consist of fibrotic tissue and may not synthesize IL-33, just as this tissue may not synthesize SP. It is not surprising that the patterns of gene expression for both SP and IL-33 are similar, as these two mediators tend to work in concert. Previous papers have reported increased expression of IL-33 in nasal polyposis and have linked this finding to increased eosinophilia in this condition (17-19). However, other research has shown that the composition of nasal polyps may differ according to genetic and environmental factors; in fact, one recent study demonstrated significantly lower eosinophilia in nasal polyps that were collected from second-generation Asian patients as compared to other ethnicities (26). It is possible that the sampled population of nasal polyps in our study manifested a lower tendency of eosinophilia, corresponding with lower levels of IL-33. The patient population sampled for analysis of nasal polyposis in this study had a high representation of Asian ethnicity; 30% of CRSwNP patients sampled for lavage fluid were Asian, and 30% of CRSwNP patients sampled for polyp tissue were Asian.

In our nasal lavage samples, we found a significantly higher amount of IL-37 in the polyp group compared to all other groups. Likewise, we saw significantly increased gene expression of IL-37 in the polyp tissue as compared to a limited number of controls. These findings may reflect the body's efforts to contain inflammation; however, our results are at odds with other reported findings of reduced IL-37 levels in nasal polyposis (24, 25). Nonetheless, a similar increase in IL-37 has been reported in other inflammatory conditions including coronary artery disease (27) and autism spectrum disorder (28). It is possible that IL-37 may play a critical regulatory role in certain types of polyps - perhaps non-eosinophilic polyps - and could represent a novel therapeutic target.

The limitations of this study include demographic differences between study groups and a limited number of normal controls. It is also difficult to completely control study groups, as there may be some overlap between patients with polyps, sinusitis, and allergy. Finally, the control tissue was collected from nasal mucosa that is different in composition from polyp tissue.

In this prospective study, we measured differential levels and gene expression of SP, IL-33, and IL-37 in the lavage fluid and nasal tissue of patients with nasal polyposis and controls. In particular, SP and IL-37 levels were elevated in the lavage fluid of patients with nasal polyps, and IL-37 had increased gene expression in the polyp tissue. This finding may help us better understand and potentially treat CRS.

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REFERENCES

1. Hopkins C. Chronic rhinosinusitis with nasal polyps. N Engl J Med 2019; 381:55-63.
2. Hulse KE, Stevens WW, Tan BK, et al. Pathogenesis of nasal polyposis. Clin Exp Allergy 2015; 45(2):328-46.
3. Patou J, Holtappels G, Affleck K, et al. Syk-kinase inhibition prevents mast cell activation in nasal polyps. Rhinology 2011; 49(1):100-6.
4. Theoharides TC, Valent P, Akin C. Mast Cells, mastocytosis, and related disorders. New Engl J Med 2015; 373(2):163-72.
5. Teranishi Y, Jin D, Takano S, Kishiko S, et al. Decrease in number of mast cells in resected nasal polyps as an indicator for postoperative recurrence of chronic rhinosinusitis. Immun Inflamm Dis 2019; 7(3):191-200.
6. Zhai GT, Li JX, Zhang XH, et al. Increased accumulation of CD30 ligand-positive mast cells associates with eosinophilic inflammation in nasal polyps. Laryngoscope 2019; 129(3):110-7.
7. Baba S, Kondo K, Suzukawa M, et al. Distribution, subtype population, and IgE positivity of mast cells in chronic rhinosinusitis with nasal polyps. Ann Allergy Asthma Immunol 2017; 119(2):120-8.
8. Patel A, Theoharides TC. Methoxyluteolin inhibits neuropeptide-stimulated proinflammatory mediator release via mTOR activation from human mast cells. J Pharmacol Exp Ther 2017; 361(3):462-71.
9. Mashagi A, Marmalidou A, Tehrani M, et al. Neuropeptide substance P and the immune response. Cell Mol Life Sci 2016; 73(22):4249-64.
10. Harrison S, Geppetti P. Substance P. Int J Biochem Cell Biol 2001; 33(6):555-76.
11. Barbosa-Cobbos RE, Lugo-Zamudio G, Flores-Estrada J, et al. Serum substance P: an indicator of disease activity and subclinical inflammation in rheumatoid arthritis. Clin Rheumatol 2018; 37(4):901-8.
12. Beatrice F, Aluffi P, Bottomicca F, et al. Nasal polyps and substance P: a preliminary report. Acta Otorhinolaryngol Ital 1994; 14(Suppl)41:35-9.
13. Drake-Lee A, Jones V, Lewin I, et al. Levels of substance P and IgE decapeptide in nasal polyp fluid and matching sera. J Laryngol Otol 1996; 110:225-7.
14. Baumgarten CR, Witzel A, Kleine-Tebbe J, et al. Substance P enhances antigen-evoked mediator release from human nasal mucosa. Peptides 1996; 17(1):25-30.
15. Lonergan A, Theoharides T, Tsilioni E, et al. Substance P and hemokinin 1 in nasal lavage fluid of patients with chronic sinusitis and nasal polyposis. OTO Open 2019; 3(3).
16. Theoharides TC, Zhang B, Kempuraj D, et al. IL-33 augments substance P-induced VEGF secretions from human mast cells and is increased in psoriatic skin. Proc Natl Acad Sci USA 2010; 107(9):4448-53.
17. Song W, Wang C, Zhou J, et al. IL-33 Expression in chronic rhinosinusitis with nasal polyps and its relationship with clinical severity. ORL J Otorhinolaryngol Relat Spec 2017; 79(6):323-30.
18. Travers J, Rochman M, Caldwell JM, et al. IL-33 is induced in undifferentiated, non-dividing esophageal epithelial cells in eosinophilic esophagitis Sci Rep 2017; 7(1):17563.
19. Tworek D, Majewski S, Szewczyk K, et al. The association between airway eosinophilic inflammation and IL-33 in stable non-atopic COPD. Respir Res 2018; 19(1):108.
20. Ozturan A, Eyigor H, Eygior M, et al. The role of IL-25 and IL-33 in chronic rhinosinusitis with or without nasal polyps. Eur Arch Otorhinolaryngol 2017; 274(1):283-88.
21. Nold MF, Nold-Petry CA, Zepp JA, et al. Interleukin 37 is a fundamental inhibitor of innate immunity. Nat Immunol 2010; 11(11):1014-22.
22. Cavalli G, Dinarello CA. Suppression of inflammation and acquired immunity by IL-37. Immunol Rev 2018; 281(1):179-90.
23. Conti P, Caraffa A, Ronconi G, et al. Impact of mast cells in mucosal immunity of intestinal inflammation: inhibitory effect of IL-37. Eur J Pharmacol 2018; 818(5):294-9.
24. Liu JX, Liao B, Yu QH, et al. The IL-37-Mex3B-Toll-like receptor 3 axis in epithelial cells in patients with eosinophilic chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2020; 145(1):160-72.
25. Cheng J, Quyang H, Du J. Expression and regulation of interleukin-37 in pathogenesis of nasal polyps. Indian

J Otolaryngol Head Neck Surg 2014; 66(4):401-6.
26. Mahdavinia M, Suh LA, Carter RG, et al. Increased non-eosinophilic nasal polyps in chronic rhinosinusitis in U.S. second-generation Asians suggests genetic regulation of eosinophilia. J Allergy Clin Immunol 2015; 135(2):576-9.
27. Li H, Shen C, Chen B, Du J, et al. Interleukin-37 is increased in peripheral blood mononuclear cells of coronary heart disease patients and inhibits inflammatory reaction. Mol Med Rep 2020; 21(1):151-60.
28. Tsilioni I, Patel AB, Pantazopoulos H, et al. IL-37 is increased in brains of children with autism spectrum disorder and inhibits human microglia stimulated by neurotensin. Proc Natl Acad Sci 2019; 116(43):21659-65.