Exploring The Relationship between FOXP3⁺ Regulatory T Cells, Eosinophilia, and Disease Severity in Chronic Rhinosinusitis with Nasal Polyps

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ABSTRACT

INTRODUCTION: Chronic Rhinosinusitis (CRS) is a common condition causing a significant worldwide burden, affecting 5%-12% of the general population. The immunoregulatory mechanisms underlying eosinophilic and non-eosinophilic CRS with polyps (CRSwNP) remain incompletely understood, particularly with respect to regulatory T cells, FOXP3⁺. **MATERIALS AND METHODS:** Using immunohistochemistry, nasal polyp tissues from eosinophilic (ECRS) and non-eosinophilic (NECRS) CRSwNP patients were examined for FOXP3⁺ Treg expression, and symptoms and disease severity measures were obtained, including on quality of life. **RESULTS:** Comparative analysis along with clinical severity assessment did not demonstrate significant differences between eosinophilic and non-eosinophilic CRSwNP in FOXP3⁺ score (5.14 vs. 4.81, p=0.453), nasal polyp score (p=0.315), SNOT-22 (p=0.510), SST (p=0.295), or VAS (p=0.182), with small to medium effect sizes (Cohen's d=0.25-0.50). Correlation analysis revealed no significant associations between FOXP3⁺ expression and clinical parameters, although SST showed a weak inverse association (ϱ= –0.335, p=0.070). Significant correlations were observed between VAS and SNOT-22 (ϱ= –0.549, p=0.0017) and between SNOT-22 and SST (ϱ= –0.470, p=0.0088), indicating consistency between subjective and objective measures. **CONCLUSION:** These findings suggest that FOXP3⁺ regulatory T cell infiltration alone may not distinguish CRSwNP endotypes, underscoring the complexity of immune regulation beyond eosinophilic status. Larger, functionally focused studies are required to further elucidate the role of Tregs in CRSwNP.

KEYWORDS: Chronic rhinosinusitis, Eosinophilic, T regulatory cells, Nasal polyps, Immunohistochemistry

INTRODUCTION

Chronic rhinosinusitis (CRS), a prevalent illness, is thought to hinder day-to-day function more severely than angina, congestive heart failure, and chronic obstructive pulmonary disease, which are considered to be less detrimental to quality of life.[1]

In CRS, phenotypes are distinguished as those without polyps (CRSsNP) and those with polyps (CRSwNP); however, regardless of the presence of polyps, both types of CRS can be either eosinophilic or non-eosinophilic, making this classification insufficient to adequately capture the disease's complex pathophysiology. Therefore, instead of focusing solely on the presence or absence of polyps, CRSwNP is now more frequently classified into eosinophilic and non-eosinophilic subtypes. Eosinophilic inflammation is considered a major pathologic hallmark of CRS.

Eosinophilic CRS (ECRS) and non-eosinophilic CRS (NECRS) were characterized by the degree of tissue eosinophilia. More severe symptoms and decreased surgical treatment efficacy are commonly linked to

ECRS.[2,3] Therefore, this patient group has been the focus of efforts to apply biological therapy, such as monoclonal antibodies. Although the threshold for tissue eosinophilia varies by country, Western nations typically use a threshold of at least 10 eosinophils per visual field.[4] Eosinophil infiltration in nasal polyps is prominent in the majority of CRSwNP patients in the Western population with Th2 cytokine dominance.[5] On the other hand, East Asian nations have been found to have higher levels of CRSwNP heterogeneity. For instance, it has been found that a Th17/Th1-dominant, neutrophilic inflammatory pattern is present in a greater number of East Asian CRSwNP patients.[6-8]

The balance between Th1 and Th2 is controlled by T regulatory (Treg) cells.[9] Recent studies indicate that Treg cells play a crucial role in the expression and regulation of T-cell subtypes in CRSwNP, both in Asian and Caucasian populations.[10] Tregs ($FOXP3^+$) play a vital role in regulating chronic airway inflammation; Forkhead box P3 (FOXP3), a master transcription factor, is crucial for the development, survival, and function of Treg cells;[11,12] however, their role in CRSwNP subtypes remains incompletely understood. Studies indicate notable differences between ECRS and NECRS in the patterns of immune cell infiltration within the tissue microenvironment.[5] Despite increasing evidence on immune heterogeneity in CRSwNP, few studies have directly compared $FOXP3^+$ regulatory T cell expression between eosinophilic and non-eosinophilic CRSwNP, particularly in Asian populations.[13] Moreover, the relationship between $FOXP3^+$ Treg infiltration and clinical symptom severity remains poorly understood. This gap limits a comprehensive understanding of immune regulation across CRSwNP endotypes. Therefore, the aims of this study were to 1) evaluate tissue eosinophilia in nasal polyps of CRSwNP patients, 2) compare the $FOXP3^+$ expression between ECRS and NECRS groups, and 3) analyse correlations between tissue immunopathology (eosinophilia, $FOXP3^+$ Tregs) and clinical severity scores.

MATERIALS AND METHODS

Overall Design

This cross-sectional observational study was conducted following approval from the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM Code: USM/JEPeM/KK/23010133).

Study Participants

Patients aged 18 and above who had been diagnosed with CRSwNP in accordance with EPOS guidelines[13], based on their medical history and confirmed by sinus CT and nasal endoscopy, were included in the trial group.

This study included 30 patients who underwent endoscopic surgery between April 2021 and January 2024 at the Department of Otorhinolaryngology, Head and Neck Surgery, Hospital Pakar Universiti Sains Malaysia (HPUSM). This was a single-centre, preliminary study. Written informed consent was obtained from all participants prior to enrolment.

Exclusion criteria for the cases included immunodeficiency, pregnancy, coagulation disorders, eosinophilic granulomatosis with polyangiitis (Churg–Strauss syndrome), or cystic fibrosis. Patients with a history of systemic (oral) corticosteroid use within four weeks prior to surgery were also excluded. For this study, participants with CRS were required to undergo a corticosteroid wash-out period prior to sample collection. Systemic corticosteroids were discontinued for at least four weeks, while intranasal corticosteroids were

withheld for a minimum of two weeks before enrolment. This wash-out period was implemented to minimise steroid-induced immunomodulation, particularly its effects on regulatory T-cell activity and downstream cytokine expression, including interleukin-35 (IL-35). Although IL-35 has a short circulating half-life, corticosteroids exert prolonged effects on immune regulation. They promote regulatory T-cell expansion and suppress type 2 and Th17 inflammatory pathways. Thus, a wash-out period based on immune recovery rather than cytokine clearance was considered necessary. The selected duration aligns with prior CRS immunology studies assessing cytokine profiles and regulatory immune responses Clinical Assessment: Symptom severity was assessed using validated questionnaires. The Sino-Nasal Outcome Test-22 (SNOT-22) was used to evaluate sinonasal symptom burden and quality of life, with scores ranging from 0 to 110, where higher scores indicate worse quality of life[14] and Visual Analog Scale (VAS) was used to asses for primary symptoms (nasal obstruction, rhinorrhoea, facial pain/pressure, and diminished sense of smell) with scores range from 0 (not troublesome) to 10 (worst imaginable).[15] The endoscopic evaluation grades the presence of nasal polyps, ranging from 0 to 4, with higher scores indicating larger polyp burden.[16] The Sniffin' Sticks test is used to evaluate olfaction level (TDI): T: Threshold, D: Discrimination, I: Identification (Total Score: 0-48).[17]

Tissue Collection and Immunohistochemistry

The sinus mucosa tissue samples used in this study were collected during surgery under general anaesthesia from the osteomeatal complex region (specifically the uncinate process). This area is consistently affected by inflammatory changes in CRS nasal polyps. It is easily accessible during endoscopic sinus surgery, making it a commonly selected site for inflammatory assessment in CRS studies.

Haematoxylin and eosin (H&E) staining was performed as the standard method for histopathological evaluation. The number of eosinophils was measured by light microscopy (original magnification, 400×, averaged over five high-power fields) independently by two authors who were blinded to the clinical data. Eosinophil counts were used to classify patients into two groups: Eosinophilic CRSwNP (ECRS;≥10 eosinophils per high-power field) and Non-eosinophilic CRSwNP (NECRS;<10 eosinophils per high-power field).

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were sectioned at a thickness of 2-4 μm and mounted on salinized glass slides. After deparaffinizing the sections, they were exposed to EnVision Flex Target Retrieval Solution (High pH 9.0) and antigen retrieval utilizing the Dako PT Link module for 20 minutes at 97°C. After antigen retrieval, the slides were treated for one hour at room temperature with a 1:500 dilution of an anti-FOXP3 monoclonal antibody (Abcam, clone 236A/E7). This antibody dilution was selected based on prior optimization to ensure specific nuclear staining with minimal background. Following the primary antibody incubation, the sections were rinsed for five minutes in distilled water and then for five minutes in Tris-buffered saline (TBS). For five minutes, the EnVision Flex Peroxidase Blocking reagent was used to stop endogenous peroxidase activity. After that, the slides were left at room temperature for 20 minutes to incubate with the secondary antibody solution. After washing in tap water and rinsing in distilled water for five minutes, immunoreactivity was detected using 0.05% diaminobenzidine (DAB) as the chromogen. The sections were then counterstained with haematoxylin, mounted with coverslips using a mounting solution, and examined under a light microscope.

Eosinophil counting and FOXP3⁺ scoring

Using H&E-stained sections, two authors independently and blindly counted the number of infiltrating eosinophil cells in five randomly selected fields under light microscopy at high magnification (400x) to assess the extent of eosinophil infiltration in the tissues. A semi-quantitative combined scoring approach, as outlined in previous studies, was employed to determine FOXP3⁺ Treg expression [18]. The staining intensity and the proportion of positively stained cells were evaluated after the complete tissue section was viewed at both low and high magnifications. Staining intensity was graded 0 (no staining), 1(weak staining), 2 (moderate staining), and 3 (significant staining). The proportion of positive cells was scored 0 (no positive cells), 1 (1-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%), and 5 (76-100%) positive cells. The intensity and percentage ratings were added up to determine the final combined score, which ranged from 0 to 8. Higher overall scores indicated a greater expression of FOXP3⁺ regulatory T cell expression within the tissue.

Data Analysis

All statistical analyses were performed using GraphPad Prism 10.5.0 (GraphPad Software, San Diego, CA, USA). To assess differences in clinical symptom scores and compare FOXP3⁺ Treg cell expression between groups with eosinophilic chronic rhinosinusitis (ECRS) and those with non-eosinophilic chronic rhinosinusitis (NECRS), an unpaired t-test was used for between-group comparisons. Data were assessed for approximate normality prior to parametric testing. Spearman's rank correlation analyses were performed to determine associations between FOXP3⁺ regulatory T cell expression and clinical severity measures, given the semi-quantitative nature of immunohistochemical scoring and the modest sample size. Effect sizes were calculated using Cohen's d to quantify the magnitude of between-group differences. Statistical significance was defined as a p-value <0.05.

RESULTS

Demographics and Clinical Characteristics of the Patients

A total of 30 CRSwNP patients were enrolled in this study, consisting of 15 patients with eosinophilic chronic rhinosinusitis (ECRS) and 15 patients with non-eosinophilic chronic rhinosinusitis (NECRS). The demographic and clinical characteristics of the participants are detailed in Table I.

Table I: Demographic analysis and its results

Variable	ECRS (n=15)	NECRS (n=15)	p-value
Age, years (mean ± SD)	50.1 ± 15.0	53.7 ± 15.4	0.514 (t-test)
Gender, n (%)	Male: 9 (60.0%) Female: 6 (40.0%)	Male: 11 (73.3%) Female: 4 (26.7%)	0.699 (Chi-square)
Age group (years)	ECRS (n=15)	NECRS (n=15)	
<30	2 (13.3%)	1 (6.7%)	
30–49	3 (20.0%)	4 (26.7%)	
50–69	8 (53.3%)	8 (53.3%)	
≥70	2 (13.3%)	2 (13.3%)	

Comparative statistical analysis between ECRS and NECRS

Comparative statistical analysis was performed using an independent t-test in GraphPad Prism 10.5.0 to assess differences in clinical, histopathological, and immunohistochemical parameters between eosinophilic chronic rhinosinusitis (ECRS) and non-eosinophilic chronic rhinosinusitis (NECRS). The summary of the mean values, p-values, and effect sizes is presented in Table II, and the comparative bar graphs are illustrated in Figure 1. With a mean FOXP3⁺ score of 5.14 in the ECRS group and 4.81 in the NECRS

group (p=0.453, Fig. 1(A), Cohen's d=0.28), indicating a small effect size. These findings were supported by representative immunohistochemical staining patterns of FOXP3⁺ regulatory T cells in ECRS and NECRS tissues (Figures 2 & 3).

Similarly, there was no significant difference (p=0.313, Fig. 1(B), d=0.27, modest impact) in the nasal polyp (NP) score between ECRS (mean 1.67) and NECRS (mean 1.93). SNOT-22 scores, which measure the burden of sinonasal symptoms, were somewhat higher in ECRS (31.73) than in NECRS (27.87), but the difference was not statistically significant (p=0.510, Fig. 1(C), d=0.25, minor impact).

Sniffin' Sticks Test (SST) scores, which measure olfactory function, were higher in NECRS (28.17) than in ECRS (24.53). Although this difference was not statistically significant (p=0.295, Fig. 1(D), d=0.39, indicating a small to moderate effect). The Visual Analogue Scale (VAS) score, which measures overall symptom severity, was higher in ECRS (mean 5.47) than in NECRS (mean 3.87), with a moderate effect size (d=0.50) and a p-value of 0.182 (Fig. 1E).

Table II: Unpaired t-test results for ECRS vs NECRS

Parameter	ECRS Mean	NECRS Mean	p-value	Cohen's d	Effect Size Interpretation
FOXP3⁺ score	5.14	4.81	0.453	0.28	Small effect
NP score	1.67	1.93	0.315	0.37	Small effect
SNOT-22	31.73	27.87	0.51	0.25	Small effect
SST	24.53	28.17	0.295	0.39	Small–moderate effect
VAS	5.47	3.87	0.182	0.5	Medium effect

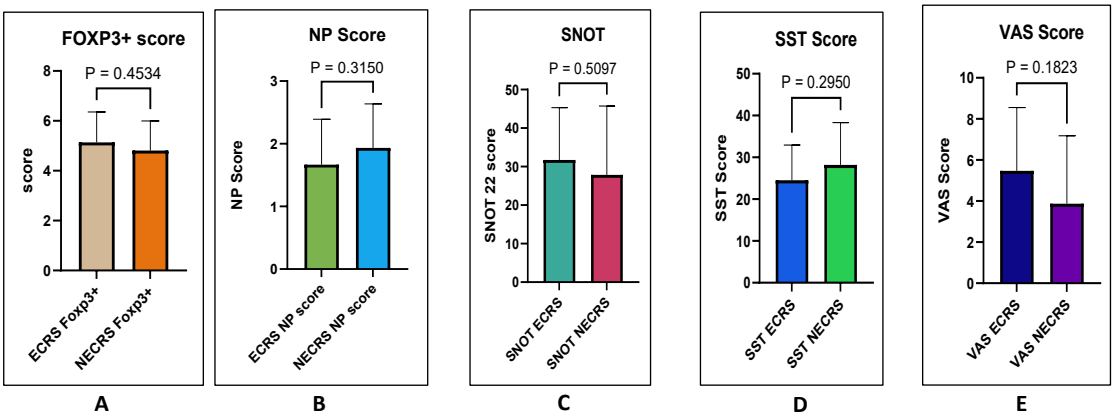


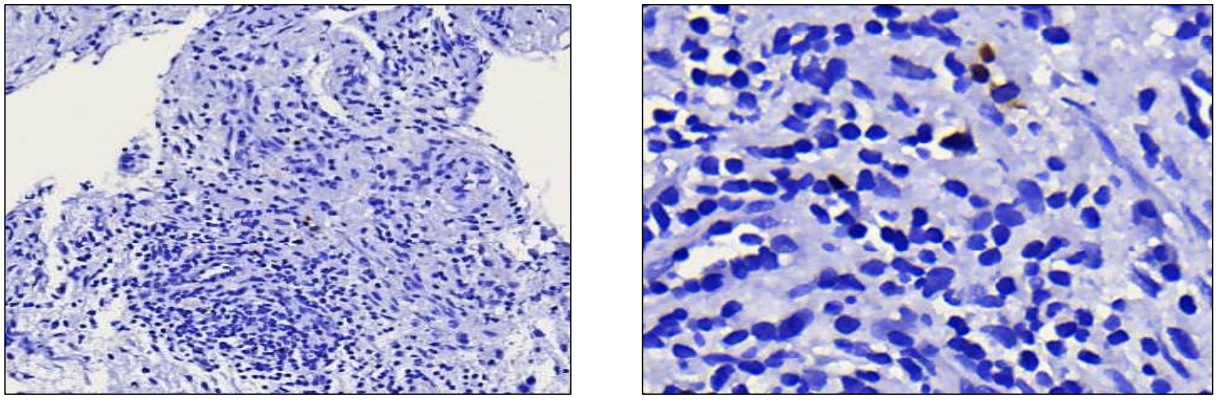
Figure 1: Comparative analysis of clinical, histopathological, and immunohistochemical parameters between eosinophilic (ECRS) and non-eosinophilic (NECRS) chronic rhinosinusitis with nasal polyps. Bar graphs represent mean ± standard deviation (SD) of (A) FOXP3⁺ score, (B) nasal polyp (NP) score, (C) SNOT-22 score, (D) Sniffin' Sticks Test (SST) score, and (E) Visual Analogue Scale (VAS) score. Statistical comparison between ECRS and NECRS groups was performed using an unpaired t-test in GraphPad Prism 10.5.0. No statistically significant differences were observed for any of the measured parameters (p>0.05). Error bars indicate SD.

Correlation analysis of clinical and immunohistochemical findings

Spearman correlation analysis was performed to evaluate the relationships among key clinical and immunological parameters listed in Table III, as illustrated in the correlation graph in Figure 4. The Visual Analog Scale (VAS) and Sinonasal Outcome Test-22 (SNOT-22) scores showed a strong negative connection (ϱ= -0.55, p =0.0017), suggesting that worse quality-of-life outcomes were associated with higher symptom load. Similarly, a strong negative association was found (ϱ= -0.47, p=0.0088) between

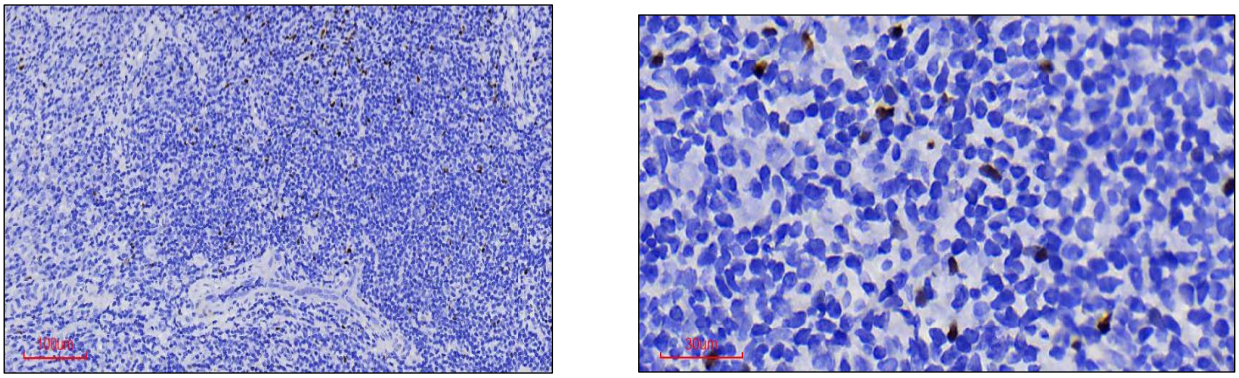
SNOT-22 and Sniffin' Sticks Test (SST) scores, indicating that higher subjective symptom scores were associated with greater olfactory impairment as measured clinically.

A borderline negative correlation was identified between FOXP3$^+$ expression and SST (ϱ= -0.34, p=0.07), reflecting a possible trend in which reduced olfactory performance is associated with higher FOXP3$^+$ cell infiltration, particularly in NECRS. However, this association did not reach statistical significance.



(A) **(B)**

Figure 2: (A) Immunohistochemical analysis of a ECRS nasal polyps tissue T-cells stained with monoclonal anti-FOXP3 antibody, Abcam [236A/E7] using 1:500 dilution under x100 magnification. (B) Immunohistochemical NECRS nasal polyps tissue labelling T-cells stained with monoclonal anti-FOXP3 antibody, Abcam [236A/E7] using 1:500 dilution under x400 magnification.



(A) **(B)**

Figure 3: (A) Immunohistochemical analysis of a NECRS nasal polyps tissue Ttissue T-cells stained with monoclonal anti-FOXP3 antibody, Abcam [236A/E7] using 1:500 dilution under x100 magnification. (B) Immunohistochemical NECRS nasal polyps tissue labelling T-cells stained with monoclonal anti-FOXP3 antibody, Abcam [236A/E7] using 1:500 dilution under x400 magnification.

Table III: Correlation analysis of immunological and clinical parameters

Pair	Spearman ϱ	p-value
FOXP3$^+$ vs NP Score	0.211	0.264
FOXP3$^+$ vs VAS	-0.088	0.644
FOXP3$^+$ vs SNOT22	0.174	0.358
FOXP3$^+$ vs SST	-0.335	0.070
NP Score vs VAS	0.180	0.342
NP Score vs SNOT22	-0.081	0.670
NP Score vs SST	-0.147	0.440
VAS vs SNOT22	-0.549	0.0017
VAS vs SST	-0.252	0.179
SNOT22 vs SST	-0.470	0.0088

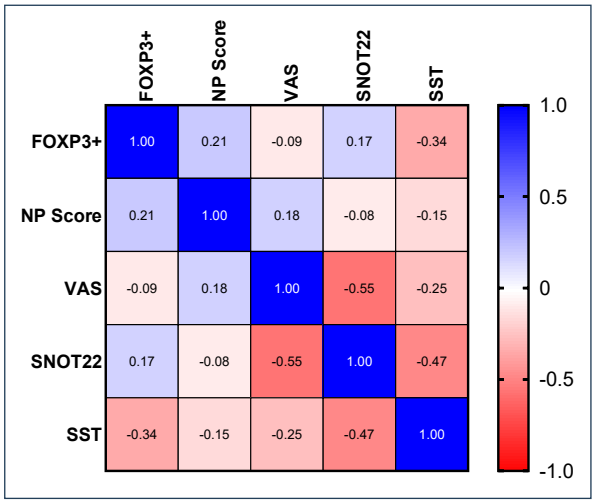


Figure 4: This heat map illustrates a significant correlation among various parameters: **VAS vs SNOT22** (ϱ= -0.55, p=0.0017) and **SNOT22 vs SST** (ϱ= -0.47, p=0.0088). **Borderline correlation**: FOXP3$^+$ vs SST (ϱ= -0.34, p=0.07) → trend, but not significant. All other correlations are weak and not significant.

DISCUSSION

The age and gender distribution of the eosinophilic (ECRS) and non-eosinophilic (NECRS) chronic rhinosinusitis groups in the current study were similar, and no statistically significant differences were found. In line with the recognised peak occurrence of CRS in this age range, the majority of patients in both groups were middle-aged to older adults (50–69 years).[18] The predominance of male patients in both groups is consistent with previous epidemiological reports indicating a higher prevalence of CRS, particularly the non-eosinophilic subtype, among men.[19] The demographic comparability between groups strengthens the internal validity of this study and minimises potential confounding effects on immunological and clinical comparisons.

This study demonstrated no statistically significant differences in FOXP3$^+$ expression, nasal polyp (NP) scores, or clinical indices, such as the SNOT-22, VAS, and olfactory function (as measured by the SST), between eosinophilic chronic rhinosinusitis (ECRS) and non-eosinophilic chronic rhinosinusitis (NECRS). The ECRS group had a slightly higher mean FOXP3$^+$ score (5.14) than the NECRS group (4.81 this difference did not reach statistical significance (p=0.453, Cohen's d=0.28). These findings suggest that FOXP3$^+$ Tregs are present across CRSwNP endotypes, regardless of eosinophilic status, and that quantitative differences alone may not be sufficient to explain disease heterogeneity.

Previous studies have established that Tregs play a crucial role in maintaining mucosal immune tolerance and that their dysregulation exacerbates chronic inflammation in CRS. Persistent exposure to type 2 cytokines may affect the function of FOXP3$^+$ Tregs;[19] however, the present findings indicate that their tissue abundance is not necessarily reduced in eosinophilic CRS.

Importantly, the functional capacity of FOXP3$^+$ T regs may be more relevant than their numerical presence. In ECRS, long-term exposure to IL-5, IL-13, and IL-33 may inhibit Treg-mediated regulation or alter their cytokine secretion profile, thereby limiting their anti-inflammatory effects. [20,21] Conversely, in NECRS, Th1- and Th17-associated cytokines such as IFN-γ and IL-17A may destabilize FOXP3 expression, reducing suppressive capacity or promoting phenotypic plasticity toward pro-inflammatory states.[22] Thus,

the absence of significant quantitative differences in FOXP3+ Cells between ECRS and NECRS likely reflects functional heterogeneity rather than immunological equivalence.

In terms of clinical outcomes, there were no discernible differences between the groups, although ECRS had slightly higher SNOT-22 and VAS ratings. This pattern is consistent with research, which has found that although individual variability can mask statistical significance in moderate-sized cohorts, eosinophilic inflammation is frequently associated with more severe symptoms and mucosal remodelling.[23,24] These findings underscore the multifactorial nature of symptom expression in CRS, where immunological patterns do not always directly translate into clinical severity.

The correlation analysis provides further insight into the clinical and immunological interactions. Increased symptom burden is associated with lower olfactory performance and a lower quality of life, according to the substantial negative correlations between VAS and SNOT-22 (ϱ= –0.55, p=0.0017) and between SNOT-22 and SST (ϱ= –0.47, p= 0.0088). The significance of SNOT-22 and VAS as supplemental clinical instruments is confirmed by these associations, which are well-documented in CRS literature.[18,25] These associations reinforce the complementary value of subjective and objective clinical assessment tools in CRS evaluation.

A borderline negative correlation (ϱ= –0.34, p=0.07) between FOXP3+ expression and SST suggests a potential trend in which increased FOXP3+ infiltration is associated with decreased olfactory function. Although not statistically significant, this observation may reflect compensatory accumulation of regulatory T cells in regions of heightened inflammation.[26] Such accumulation may coexist with epithelial damage, neuronal loss, and impaired mucosal regeneration, ultimately contributing to olfactory dysfunction.[27] Larger studies are required to clarify whether this trend represents a biologically meaningful relationship.

Overall, these results highlight that the CRS pathophysiology extends beyond a simple dichotomy of eosinophilic versus non-eosinophilic inflammation, but rather a complex interplay between regulatory and effector immune pathways. Molecular endotyping of CRS reveals several overlapping inflammatory profiles that are not strictly phenotype-dependent, as noted.[28] Therefore, future studies integrating FOXP3+ expression with functional assays and multiplex cytokine analyses including IL-10, IL-35, and TGF-β may provide deeper insights into the immunoregulatory mechanisms underlying CRS.

This study has several limitations. The relatively small sample size may have limited the statistical power to detect subtle differences between CRS subtypes. Additionally, FOXP3+ expression was assessed using immunohistochemical scoring, which reflects cellular presence but does not directly measure functional suppressive activity. The cross-sectional design also precludes causal inference. Future studies incorporating larger cohorts and functional immunological assays are warranted to further clarify the role of regulatory T cells in CRS endotypes.

CONCLUSION

Previous studies determining the variability in tissue infiltration by immune cells have reported heterogeneous findings. The present study contributes to the growing body of evidence by examining FOXP3+ regulatory T-cell expression and its clinical correlations across eosinophilic and non-eosinophilic CRSwNP endotypes. Although no statistically significant differences were observed in FOXP3+ expression

or clinical severity scores between ECRS and NECRS, the observed trends underscore the complexity of immune regulation in CRS.

These findings suggest that CRS pathophysiology cannot be explained solely by eosinophilic status and likely reflects overlapping and dynamic immunoregulatory mechanisms involving both regulatory and effector immune pathways. Notably, the absence of significant quantitative differences highlights the potential relevance of functional immune modulation rather than immune cell abundance alone.

Future studies with larger cohorts and integrated functional and molecular analyses are warranted to elucidate endotype-specific immunoregulatory mechanisms further and to support the development of targeted therapeutic strategies for CRS.

FUNDING

The writing and research to produce this manuscript were funded by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme (Project code: FRGS/1/2022/SKK06/USM/02/5)

CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

INSTITUTIONAL REVIEW BOARD (ETHIC COMMITTEE)

This cross-sectional observational study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM Code: USM/JEPeM/KK/23010133) before the study. Written informed consent was obtained from all participants prior to inclusion in the study. Participants were informed about the study objectives, procedures, and their right to withdraw at any time without penalty.

CONSENT FOR PUBLICATION

Written informed consent for publication of the clinical data and findings was obtained from all participants included in this study.

AUTHOR'S CONTRIBUTION

Conceptualisation: NMS, GG, Supervision: NMS, NACJ. Data curation: GG, NMS, FSM. Formal analysis: GG, NACJ, RK. Funding acquisition: NMS. Methodology: GG, NMS, ATMY. Project administration: NMS, GG, RRR. Visualisation: GG, NMS, NMA. Writing - original draft: GG, NMS. Writing - review & editing: NMS, GG, BA.

ACKNOWLEDGMENTS

We would like to thank the Ministry of Higher Education for the research grant provided (FRGS/1/2022/SKK06/USM/02/5) and thank the staff members of the Pathology Laboratory Unit at Hospital Pakar Universiti Sains Malaysia for their assistance.

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