



Neutrophil infiltrates and eosinophil aggregates in chronic rhinosinusitis with nasal polyps and EGPA

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Abstract

Introduction/objectives The histopathological study of inflammatory cells and their tendency to form aggregates in chronic rhinosinusitis with nasal polyps (CRSwNP) has shown promising results in determining the pathogenesis and predicting clinical outcome. Bilateral nasal polyps also occur in over 70% of patients with eosinophilic granulomatosis with polyangiitis (EGPA). The study aim was to investigate neutrophil infiltrates and eosinophil aggregates in CRSwNP and EGPA tissues of Caucasian patients.

Method A histopathological study was performed on surgical specimens of nasal polyps from 144 adults (15 with allergic fungal rhinosinusitis; 19 with nonsteroidal anti-inflammatory drugs-exacerbated respiratory disease (NERD); 16 with intrinsic asthma; 21 with extrinsic asthma; 21 with allergy; 22 with eosinophil CRSwNP (ECRSwNP); 17 with non-ECRSwNP; 13 with EGPA).

Results Focusing on the presence of tissue eosinophil aggregates, NERD and ECRSwNP were the sub-cohorts with the highest rate. Neutrophil infiltrate rate was significantly higher in EGPA sub-cohort than in all CRSwNP sub-cohorts apart from non-ECRSwNP.

Conclusions Structured histopathology is increasingly identifying the different histotypes of CRSwNP. This analysis can be used to better understand CRSwNP endotypes and develop targeted therapies. The response to therapy and therefore control of CRSwNP relapses definitely depends on our ability to act on the underlying inflammatory pattern.

Key points

- Systematic analysis of how neutrophil infiltrates and eosinophilic aggregates are distributed in the different phenotypes of CRSwNP and EGPA.
- Neutrophil infiltrates and eosinophil aggregates are strong risk factors for nasal polyps' refractoriness.
- NERD and ECRSwNP are the sub-cohorts of CRSwNP with the highest rate of tissue eosinophil aggregates.
- Neutrophil infiltrates are significantly higher in EGPA.

Keywords Chronic rhinosinusitis with nasal polyps · Eosinophil aggregates · Eosinophilic granulomatosis with polyangiitis · Neutrophil infiltrates · Structured histopathology

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Introduction

Chronic rhinosinusitis (CRS) is a significant multifactorial airway disease affecting 5–12% of the general population with different prevalence around the world: 7 to 27% in Europe, 12% in the USA, and 8% in China [1–3]. CRS is divided in two phenotypes: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP). Several studies supported the hypothesis that distinct immunological mechanisms characterize both CRSsNP and CRSwNP, allowing identification of the different endotypes [4–8].

Based on the tissue eosinophil count, CRSwNP is classified into eosinophil (ECRSwNP) and non-eosinophil (non-ECRSwNP) subtypes [9]. ECRSwNP is mainly characterized by Th2 response, whereas the non-ECRSwNP by Th1 and Th17 activation. Inflammatory patterns change across geographical areas. ECRSwNP is more common in western countries, whereas non-ECRSwNP is prevalent in Asia [5, 10, 11]. Eosinophil inflammation is a quite well-established feature that is associated with higher recurrence rates after treatment than non-eosinophil forms [12]. Neutrophil inflammation remains less defined. Several studies revealed that increased neutrophils in CRSwNP tissue reduce response to oral corticosteroid therapy [13, 14] and play an important role in nasal polyps' relapse [15].

In eosinophilic granulomatosis with polyangiitis (EGPA), more than 70% of patients suffer from CRS with diffuse and bilateral nasal polyps: these polyps are very similar to those of ECRSwNP, characterized by severe eosinophil infiltration with a chronic-relapsing course despite surgery and medical treatment in almost one-third of cases [16, 17]. Our research group recently studied the histopathological features of nasal polyps in EGPA patients, finding an abundance of neutrophils in surgical specimens [18] and supporting the hypothesis of an involvement of neutrophil infiltration in disease recurrence.

Neutrophil infiltrates and eosinophil aggregates seem to affect CRSwNP patients' outcome although studies regarding these features in nasal polyps are still at an early stage. This fact highlights the need to better understand the distribution of neutrophil infiltration and its relationship with eosinophil aggregates in nasal polyps' tissue. Histopathology can provide meaningful information for this purpose [19]. The main aim of this retrospective study was to analyze how neutrophil infiltrates and eosinophil aggregates are represented in CRSwNP and EGPA tissue of Caucasian patients.

Materials and methods

Patients

The study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in agreement with the Italian privacy and sensitive data laws and internal rules of Padova University's Otolaryngology section. All patients signed a detailed informed consent form regarding the processing and publication of their data.

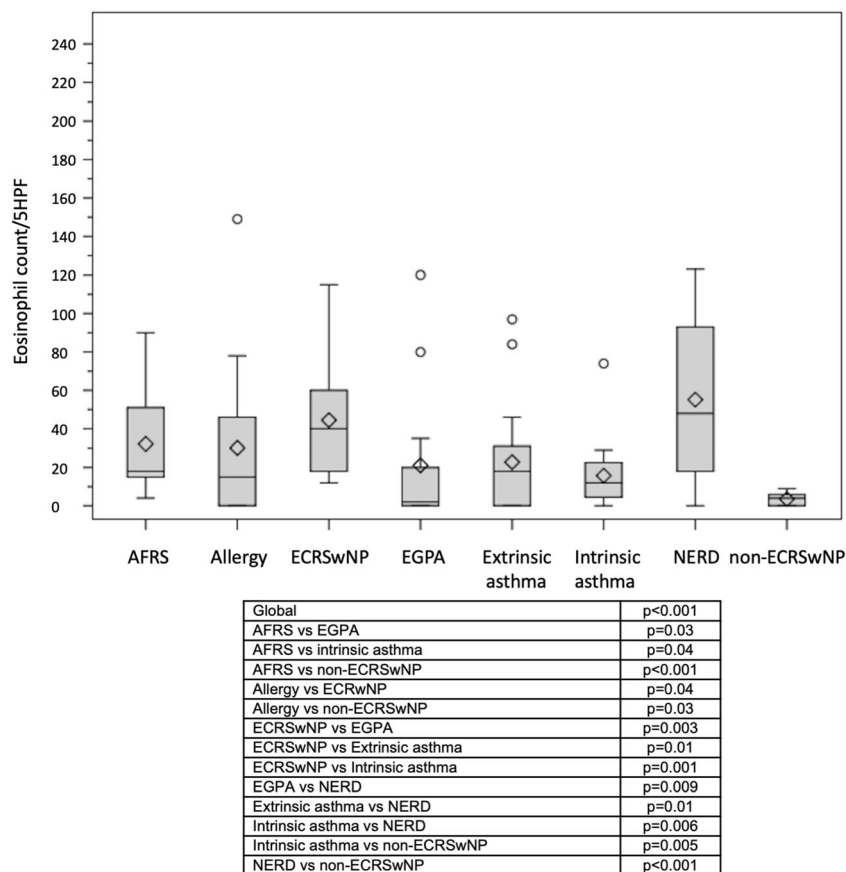
We retrospectively assessed 144 Caucasian adult patients: 131 CRSwNP patients and 13 EGPA ones with nasal polyps, who underwent primary endoscopic sinus surgery (ESS) in our Institution from 2009 to 2017. Patients with autoimmune disease other than EGPA, onco-hematological disorders, acute or chronic infectious diseases other than sinusitis, and chronic renal insufficiency or who were pregnant were excluded from

Table 1 Demographic and histopathological data regarding the sub-cohorts of patients considered

		NERD (No. = 19)	AFRS (No. = 15)	Allergy (No. = 21)	Extrinsic asthma (No. = 21)	Intrinsic asthma (No. = 16)	EGPA (No. = 13)	ECRSwNP (No. = 22)	Non-ECRSwNP (No. = 17)
Gender (M)	No. (%)	11 (57.9)	10 (66.7)	10 (47.6)	15 (71.4)	11 (68.8)	5 (38.5)	19 (86.4)	10 (58.8)
Age (years)	Mean (SD)	54.9 (13.4)	48.3 (13.2)	41.0 (12.8)	49.2 (16.0)	52.0 (12.1)	53.3 (12.3)	55.1 (12.6)	51.8 (16.4)
	Median (IQR)	52 (46–67)	43 (41–57)	39 (29–48)	51 (39–63)	54 (45–61)	57 (46–58)	55 (49–63)	52 (37–69)
Eosinophil count/5 HPF	Mean (SD)	55.1 (41.6)	32.1 (27.1)	30.1 (38.1)	22.8 (27.7)	15.8 (18.1)	21.1 (37.4)	44.5 (30.4)	3.5 (3.5)
	Median (IQR)	48.0 (18.0–93.0)	18.0 (15.0–51.0)	15.0 (0.0–46.0)	18.0 (0.0–31.0)	12.0 (4.5–22.5)	2.0 (0.0–20.0)	40.0 (18.0–60.0)	4.0 (0.0–6.0)
Eosinophil aggregate, presence	No. (%)	8 (42.1)	1 (6.7)	6 (28.6)	2 (9.5)	1 (6.3)	1 (7.7)	9 (40.9)	0 (0.0)
Neutrophil infiltrate, presence	No. (%)	5 (26.3)	4 (26.7)	4 (19.0)	3 (14.3)	3 (18.8)	10 (76.9)	5 (22.7)	8 (47.1)

IQR, interquartile range; HPF, high-power field; M, male; SD, standard deviation

Fig. 1 Box plots of eosinophil polyps tissue counts in the evaluated sub-cohorts. Only significant differences have been reported



this study. Information about sensitivity to acetylsalicylic acid or other nonsteroidal anti-inflammatory drugs (NSAIDs) were obtained from their medical history, recorded in Padova University Hospital's electronic archives [Galileo]. All patients underwent preoperative tests at the Laboratory Medicine Service of Padova University Hospital, concerning their total and specific IgE for *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, birch pollen, pellitory, grass mix, cat and dog dander, *Alternaria alternata*, *Aspergillus fumigatus*, and common ragweed, as previously reported [20]. The diagnosis of asthma was confirmed according to the definition of the Global Initiative on Asthma [21]. Asthma was then defined extrinsic when associated with allergy and intrinsic when triggered by nonallergic factors. The diagnosis of NSAID-exacerbated respiratory disease (NERD) was clinically established following the algorithm proposed by Kowalski et al. [22]. Allergic fungal rhinosinusitis (AFRS) was diagnosed in accordance with the diagnostic criteria proposed by Bent and Kuhn [23]. EGPA patients fulfilled the American College of Rheumatology criteria and had paranasal sinus involvement with nasal polyps. CRSwNP tissue eosinophilia has been staged as fairly standardized in western countries: ≥ 10 eosinophils per high-power field (HPF) indicate ECRSwNP and < 10 /HPF non-ECRSwNP [24].

Histopathological features

Surgical tissue samples were routinely processed, stained with hematoxylin and eosin (H&E), and evaluated by the same dedicated head and neck pathologist (L.A.). In detail, every H&E slide was examined under a light microscope at low-power magnification ($\times 40$) to identify the most representative fields for each histological feature considered. According to a previously reported methodological approach [19, 25, 26] with a magnification of $\times 100$, a semi-quantitative two-tier score was assigned according to the presence or absence of eosinophil aggregates and neutrophil infiltrate. To count the eosinophils in the sub-epithelial connective tissue, for each case, five HPFs of the most representative fields were examined at $\times 400$ magnification, and the mean of the five counts was recorded. Intraepithelial eosinophils and those inside the vessels were not counted. Moreover, we considered a neutrophil infiltrate when neutrophils count was at least 15–20 neutrophils/HPF, regardless of the specific histologic subsite (intraepithelial, connective tissue, perivascular) or the association with other inflammatory cells (i.e., eosinophils). Neutrophils associated with ulcers or surface epithelial erosions were not considered.

Statistical analysis

Results are reported as count and percentage for categorical variables and as mean and standard deviation (SD), median, and interquartile range (IQR) for quantitative variables. Normality of quantitative variables was verified in each group of patients with the Shapiro-Wilk test.

Groups of patients were compared with Fisher's exact test and with Kruskal-Wallis test in the case of categorical and quantitative variables, respectively. If statistical significance was reached, pair-wise comparisons were performed with Fisher's exact test and Wilcoxon rank sum test, respectively.

Statistical significance was set at the 5% level.

Results

Patients

The cohort of patients was distributed according to their clinical-pathological features as follows: AFRS (15 patients), NERD (19 patients), intrinsic asthma (16 patients), extrinsic asthma (21 patients), allergy (21 patients), ECRSwNP (22

patients), non-ECRSwNP (17 patients), and EGPA (13 patients).

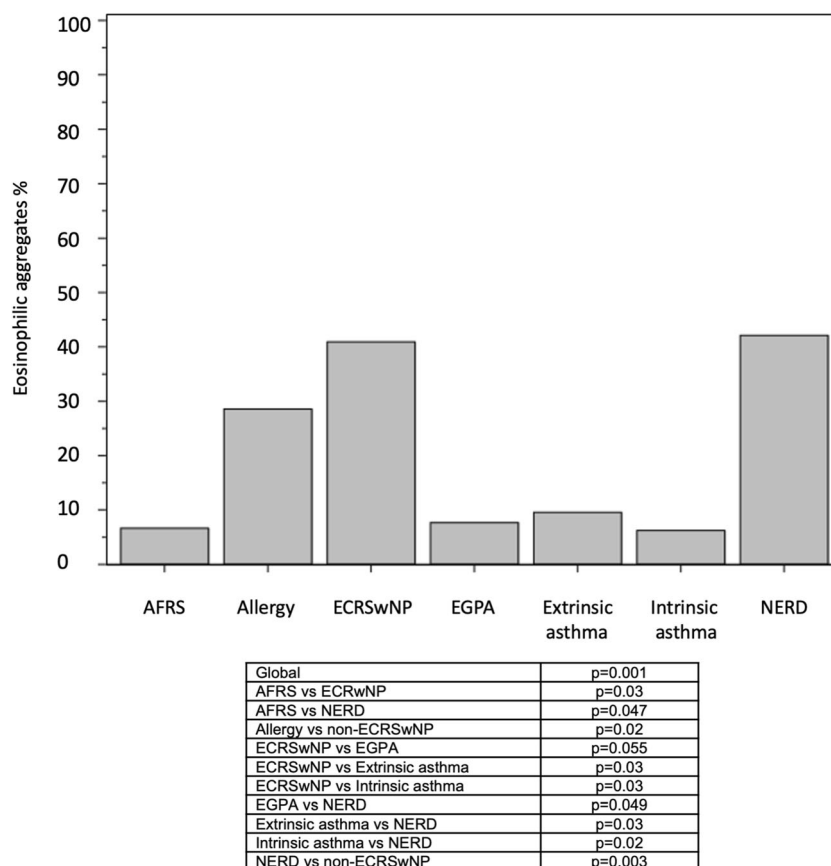
As previously reported [27], all CRSwNP patients were first preoperatively treated medically for 3 months with local mometasone furoate 200 µg daily (100 µg/nostril) or fluticasone furoate 110 µg daily (55 µg/nostril), plus oral therapy with methylprednisolone (32 mg daily on days 1–5, then 16 mg daily on days 6–10, then 8 mg daily on days 11–20). When this medical therapy failed, patients underwent ESS under general anesthesia. In EGPA sub-cohort, 7 out of 13 patients were taking oral glucocorticoids; 5 patients were also under immunosuppressive therapy. Four EGPA patients were not diagnosed before ESS and had no medical treatments; data are missing for 2 patients.

Table 1 stratifies the considered sub-cohort of patients in terms of their demographic and clinical-pathological features. The mean age of allergic patients was significantly lower than AFRS ($p = 0.004$), ECRSwNP ($p = 0.001$), intrinsic asthma ($p = 0.02$), and non-ECRSwNP ($p = 0.03$) ones.

Analysis of the histopathological features

Figure 1 shows the tissue eosinophil count values for each sub-cohort and reports the significant differences between

Fig. 2 Eosinophil aggregates presence in nasal polyps' tissue of the considered sub-cohorts. Statistically significant differences have been reported



nasal polyps considered sub-cohorts. Focusing on the presence of tissue eosinophil aggregates (Fig. 2), NERD (Fig. 3a, b, c) and ECRSwNP were the sub-cohorts with the highest rate.

As can be seen in Fig. 4, neutrophil infiltrate positive rate was significantly higher in EGPA sub-cohort (Fig. 5a, b, c) than in all CRSwNP sub-cohorts with the only exception of the non-ECRSwNP one (Fig. 6a, b). No bacterial infection-associated neutrophils were found. No CRSwNP sub-cohorts differed significantly from one another in terms of neutrophil infiltrate.

Discussion

ECRSwNP is mainly related to a type 2 immune response that induces differentiation and maturation of eosinophils. ECRSwNP has been extensively studied and associated with prognosis in terms of high recurrence rate [28]. Type 2 inflammation, characterized by a prominent role of cytokines such as IL-4, IL-5, and IL-13, is generally responsive to glucocorticoids, which are routinely used as first choice drug [7]. Several studies have recently considered not only the tissue inflammatory cells but also their tendency to form aggregates and their position with respect to vessels and stroma [29]. Kuhar et al. [26] studied 13 histopathological variables in the ethmoid sinus tissue removed from 99 patients during ESS for CRS. They reported that eosinophilic aggregates were associated with significantly worse disease, possibly signifying a unique subtype. In 2019, Tajudeen et al. [30] reviewed 101 patients who had undergone ESS for refractory CRS. After structured histopathology, they found that CRS patients with eosinophilia required more cumulative steroids to control the disease post-operatively compared to patients without eosinophilia. Patients with tissue eosinophil aggregates required the most cumulative steroids. When removing patients with eosinophil aggregates from the eosinophilia group, no difference in therapeutic response persisted between patients with eosinophilia and those without. The authors concluded that tissue eosinophil aggregates appeared to be the major driving factor for increased prednisone requirements after ESS to control the mucosal disease than mere presence of eosinophils. According to our data, eosinophilic aggregates were found to be significantly more frequent in NERD and ECRSwNP sub-cohorts. Eosinophilic aggregates were absent in non-ECRSwNP patients (see Fig. 2).

Neutrophils have traditionally been acknowledged as the first immune cells that are recruited to inflamed tissues during acute inflammation. On the contrary, their role within the context of chronic inflammation has been less studied: in particular, neutrophilic inflammation in CRS remains very poorly defined [31]. Tissue neutrophilia has been increasingly

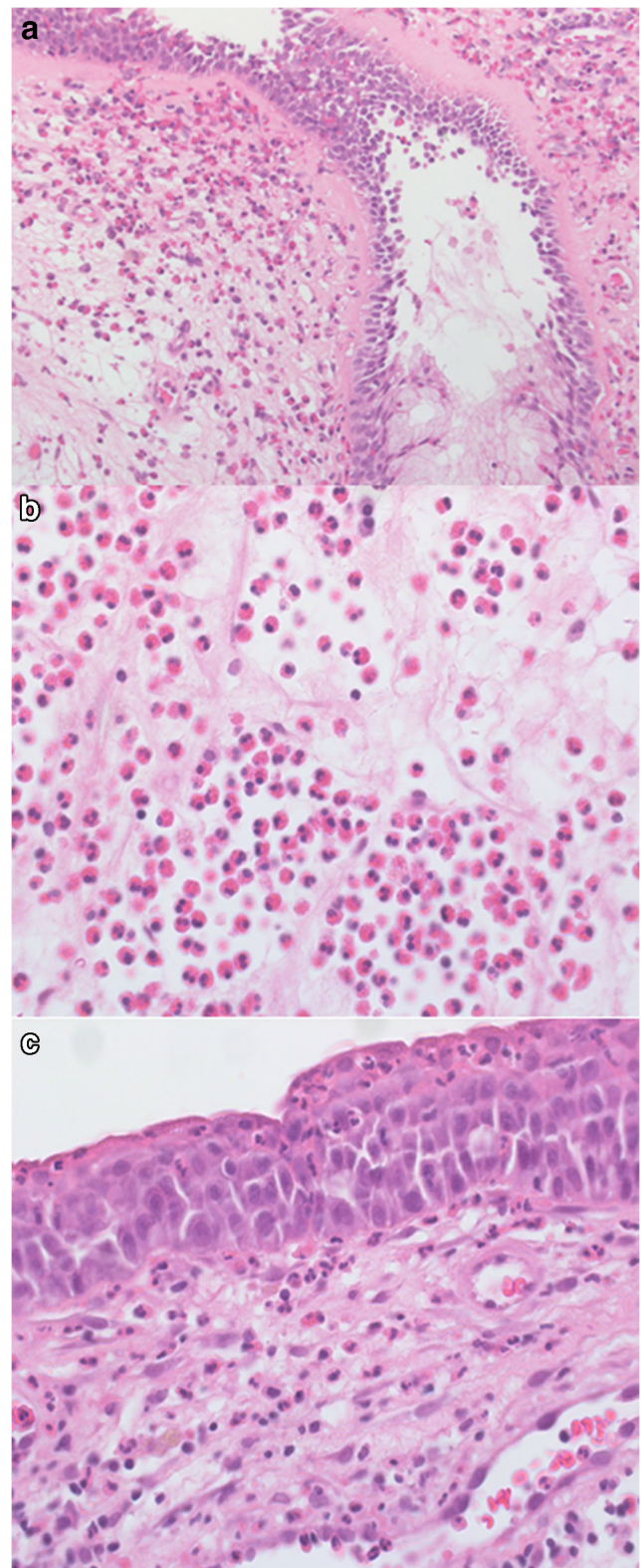
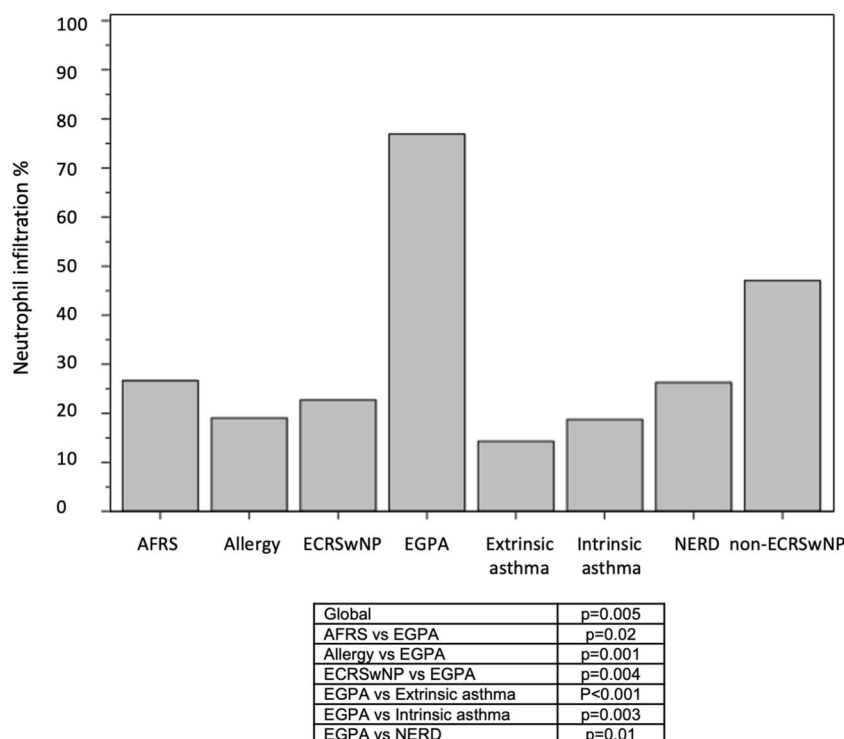


Fig. 3 A case belonging to the NERD sub-cohort, with high eosinophil count (**a** original magnification $\times 200$), diffuse presence of eosinophil aggregates (**b** original magnification $\times 400$), and rare neutrophils in the squamous metaplastic epithelium as well as in the connective tissue (**c** original magnification $\times 400$)

Fig. 4 Neutrophil infiltration presence in nasal polyps of the considered sub-cohorts. Only significant differences have been reported



recognized as a strong risk factor for CRSwNP refractoriness, assuming a crucial connection between neutrophils and difficult-to-treat forms of the disease [32, 33]. Recently, Kim et al. [32] histologically investigated 160 cases of CRSwNP (40 ECRSwNP [> 70 eosinophils/HPF] and 120 non-ECRSwNP [< 70 eosinophils/HPF]). They defined tissue neutrophilia (Neu^{high}) for cases with more than 20 human neutrophil elastase (HNE)-positive cells/HPF and the absence of tissue neutrophilia (Neu^{low}) for those with less than 20 HNE-positive cells/HPF. Stratifying patients according to these variables, the authors found that non-ECRSwNP-Neu^{low} patients showed a higher rate of disease control than non-ECRSwNP-Neu^{high} and ECRSwNP-Neu^{high} patients. Linear by linear association demonstrated the trend in refractoriness from non-ECRSwNP-Neu^{low} to non-ECRSwNP-Neu^{high} or ECRSwNP-Neu^{low} to ECRSwNP-Neu^{high}. When multiple logistic regression was performed, tissue neutrophilia was found to be the strongest risk factor for CRSwNP refractoriness. These findings suggested that some patients had increased neutrophilic inflammation along with eosinophilic inflammation. It should be remembered that a worse outcome can be due to either a significant aggressiveness of the CRSwNP or a limited response to common corticosteroid therapy. In contrast to their suppressive effect on eosinophils, pharmacological studies show that corticosteroids prevent apoptosis of neutrophils and promote the neutrophilic inflammation [31]. Our data showed the presence of neutrophil infiltrates mainly in EGPA and non-ECRSwNP patients. In EGPA

nasal polyps, we found a diffuse, rather than perivascular, neutrophilic infiltration, supporting the involvement of neutrophils in the inflammatory process, even when there were no signs of vasculitis. There is a dearth of information regarding the role of neutrophils in nasal polyps' tissue of EGPA patients. As well as exerting their typical innate anti-microbial activity, activated neutrophils can also prime a Th1- and Th17-acquired immune response and enhance eosinophil migration [34]. The role of nasal tissue neutrophil infiltrate might therefore be to amplify eosinophil tissue recruitment, in addition to contributing to inflammation [18]. Moreover, neutrophils may also be involved in lung inflammation. Interstitial neutrophil infiltrates were found in five open lung biopsies from active EGPA patients by Dong et al. [35], suggesting an active role of these cells in the pathogenesis of tissue inflammation. Neutrophils could be activated by anti-neutrophil cytoplasmic antibody (ANCA). Although a role of ANCA in EGPA is still unclear and myeloperoxidase (MPO)/ANCA antibodies are found in only 35–40% of EGPA patients, ANCA can be localized in the tissues. Mukherjee et al. [36] found ANCA in the sputum of serum ANCA-negative EGPA patients, suggesting a local autoimmune response. Immunoglobulin from ANCA+ sputa, similar to serum ANCA, was able to trigger extracellular DNA from neutrophils, a well-known mechanism in ANCA pathology [37], as well as eosinophil extracellular traps [36]. Moreover, the recent published genome-wide study demonstrated that EGPA is polygenic with genetic distinctions between MPO ANCA-

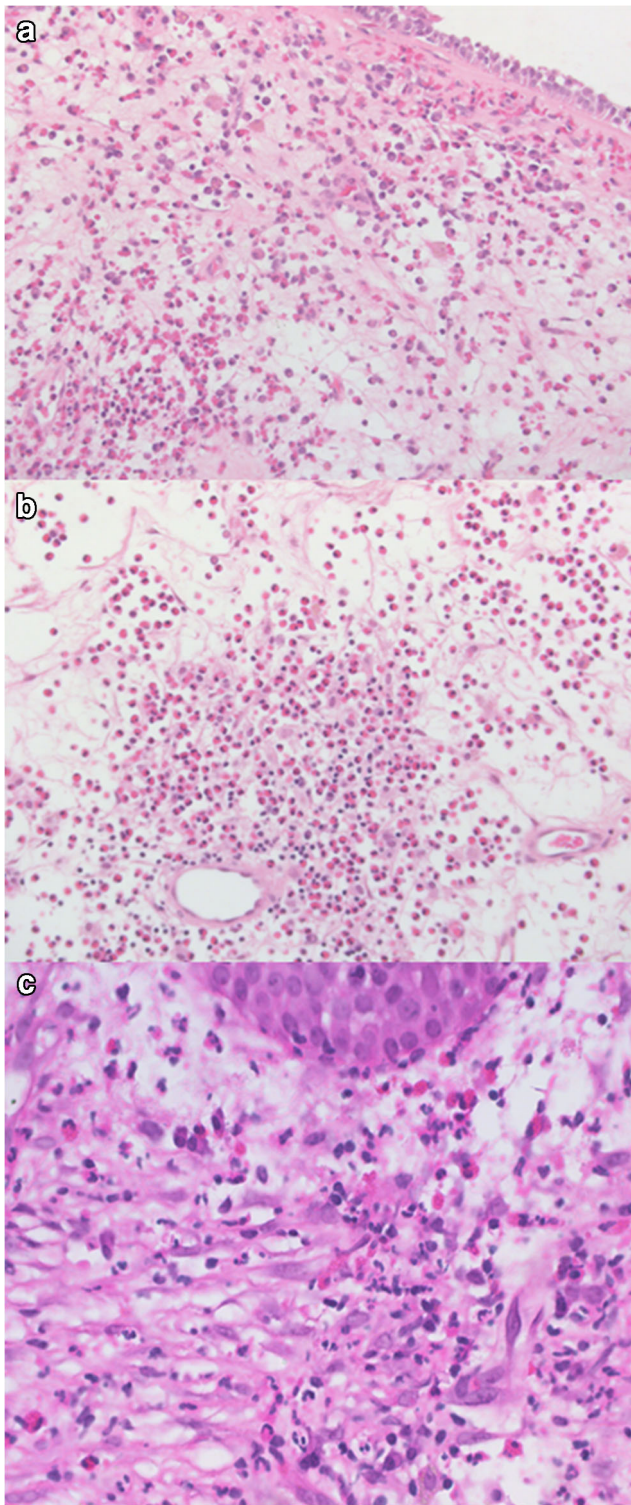


Fig. 5 An EGPA patient showing a high eosinophil count (**a** original magnification $\times 200$) along with foci of eosinophil aggregates (**b** original magnification $\times 200$) and an evident neutrophilic infiltrate in the sub-epithelial connective tissue (**c** original magnification $\times 400$)

positive and ANCA-negative disease, correlating with different clinical features. Therefore, EGPA might be comprised of two distinct diseases defined by ANCA-

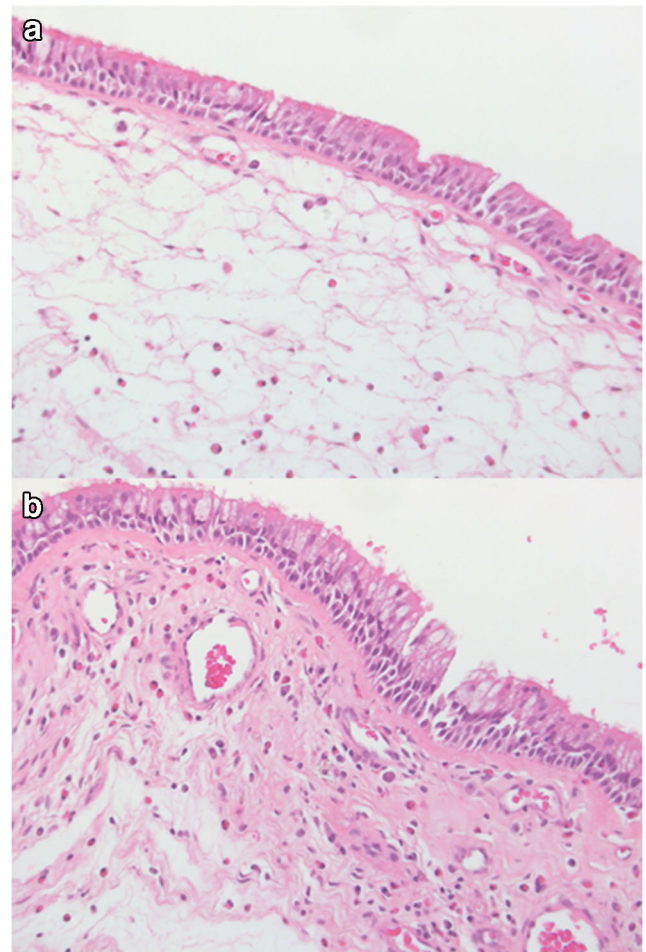


Fig. 6 The histological features of a non-ECRwNP case: very low eosinophil count (**a** original magnification $\times 200$) and absence of eosinophil aggregates (**b** original magnification $\times 200$)

negative, characterized by eosinophils tissue infiltration and a mucosal/barrier dysfunction, and MPO-positive ANCA disease, characterized by an eosinophilic autoimmune response sharing clinical features and HLA-DQ association with classic MPO + ANCA-associated vasculitis [38]. These studies hint the possibility that, yet with unknown mechanism, neutrophils and ANCA by interacting with eosinophils might play a pathogenetic role in EGPA, similarly to granulomatosis with polyangiitis and microscopic polyangiitis. Blocking the trafficking or activation of neutrophils could be hypothesized as a useful therapeutic strategy in selected CRSwNP (i.e., non-ECRSwNP). Strategies to inhibit neutrophil recruitment may include blockage of chemokine receptors (CXCR1 and CXCR2 antagonists) and inhibition of IL-36 γ , IL-17A, and LTB4 [31].

Our study has some limitations concerning the retrospective exploratory setting. Its main strength lies in the multidisciplinary setting in which all patients were diagnosed and treated, including the surgical procedures, performed by the same team of otolaryngologists, and the histopathological

analysis evaluated by the same dedicated head and neck pathologist. These analyses were not conducted on biopsies but only on surgical sections, allowing an accurate analysis of the different cells' infiltration and distribution in the tissues. Information on the structural histopathology of EGPA nasal polyps, in which our group is developing a certain experience [18, 39], is still lacking in the literature.

Structured histopathology appears increasingly interesting for rhinologists in identifying the different histotypes of CRSwNP. Knowledge of these histotypes could be used to better understand CRSwNP endotypes and develop targeted therapies. The identification of various inflammatory biomarkers (including cell lines) has led to the development of monoclonal antibodies that target the underlying mechanisms of inflammation [40]. This therapeutic approach is destined to be successful only if the inflammatory mechanisms and involved cytotypes are correctly identified. It should be emphasized that the response to therapy and therefore control of CRSwNP relapses depend exclusively on our ability to act on the inflammatory pattern responsible for the disease.

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Authors' contributions GC: conceptualization, investigation, writing – review and editing; GB: conceptualization, investigation, writing – review and editing, supervision ENT topics; LA: investigation, writing – review and editing, supervision pathological topics; UB: review and editing, supervision ENT topics; RP: review and editing, supervision rheumatologic topics; ACF: statistical analysis, writing – review and editing; FS: review and editing, supervision rheumatologic topics; GM: conceptualization, investigation, writing – review and editing, supervision ENT topics, funding acquisition.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Disclosures None.

Ethics approval The study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in agreement with the Italian privacy and sensitive data laws and internal rules of Padova University's Otolaryngology section.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication Patients signed informed consent regarding publishing their data and photographs.

Code availability N/A.

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