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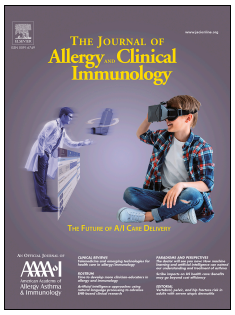

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Tim Delemarre, MSc, Gabriele Holtappels, BSc, Natalie De Ruyck, MSc, Nan Zhang, MD PhD, Hans Nauwynck, DVM PhD, Claus Bachert, MD PhD, Elien Gevaert, PhD

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A substantial neutrophilic inflammation as regular part of severe type 2 chronic rhinosinusitis with nasal polyps

Tim Delemarre MSc[1], Gabriele Holtappels BSc[1], Natalie De Ruyck MSc[1], Nan Zhang MD PhD[1], Hans Nauwynck DVM PhD[2], Claus Bachert MD PhD[1,3]*, Elien Gevaert PhD[1]*

Affiliations:

[1] Upper Airways Research Laboratory, Faculty of Medicine, Ghent University, Ghent, Belgium;

[2] Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium;

[3] Division of ENT Diseases, CLINTEC, Karolinska Institute, Stockholm, Sweden

***** Claus Bachert and Elien Gevaert shared project supervision

Corresponding author:

Claus Bachert, MD, PhD; Upper Airways Research Laboratory, Department Head & Skin, Ghent University; UZ Ghent, C. Heymanslaan 10, 9000 Ghent, Belgium.

E-mail: Claus.Bachert@ugent.be

Phone number: +329 332 2363

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Abstract (250 words)

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is generally associated with severe type 2 immune reactions in the Caucasian population. However, recent findings suggest an additional role for neutrophils in severe type 2 inflammation.

Objective: We aimed to characterize the neutrophilic inflammation in CRSwNP and its relation to eosinophilic inflammation in severe type 2 immune reactions.

Methods: The presence and activation of neutrophils and eosinophils was analyzed in CRSsNP and CRSwNP by measuring cell and activation markers via immunohistochemistry, immunofluorescence, Luminex assay, ELISA, UniCAP, FACS and PCR. Differential neutrophil migration was assessed via Boyden-chamber assay and neutrophil survival was analyzed via flow cytometry.

Results: Both CRSsNP and CRSwNP displayed variable degrees of eosinophilic and neutrophilic inflammation, with a profound neutrophilic infiltration and activation in type 2 CRSwNP, associated with EETosis and Charcot-Leyden crystals (CLCs), but independent of IL-17. NETosis in CRSwNP was associated with bacterial colonization, however, neutrophils were less prone to undergo NETosis in the tissue of severe type 2 CRSwNP patients. Neutrophils did not show increased migration nor survival in the CRSwNP environment *in vitro*.

Conclusion: We here demonstrated a severe neutrophilic inflammation associated with severe eosinophilic type 2 inflammatory CRSwNP, the role of which needs further study.

Key Messages

- Type 2 CRSwNP displays a severe neutrophilic inflammation, characterized by increased neutrophil infiltration and activation.
- The presence of Charcot-Leyden crystals is associated with neutrophilic infiltration in CRSwNP.
- Neutrophils are less prone to generate NETs in CRSwNP tissue and NETosis in CRSwNP is associated with bacterial colonization.

Capsule Summary

We show that severe type 2 CRSwNP patients display a profound neutrophilic inflammation, with increased activation status and proteolytic activity, co-existing with severe eosinophilia (EETosis and CLC-deposition), and independent of IL-17.

Keywords:

CRSsNP, CRSwNP, type 2 inflammation, neutrophils, NETosis, Interleukin-17, Charcot-Leyden crystals.

List of Abbreviations

CRS: Chronic rhinosinusitis

CRSsNP: Chronic rhinosinusitis without nasal polyps

CRSwNP: Chronic rhinosinusitis with nasal polyps

IT: inferior turbinate

IL: Interleukin

EETosis: Eosinophil extracellular traps cell death

NETosis: Neutrophil extracellular traps cell death

MPO: Myeloperoxidase

ECP: eosinophil cationic protein

MBP: Major basic protein

NE: neutrophilic elastase

CitH3: Citrullinated histone 3

Gal10: Galectin-10

CLC: Charcot-Leyden crystal

IgE: Immunoglobulin-E

S. aureus: *Staphylococcus aureus*

1. Introduction

Chronic rhinosinusitis (CRS) is a chronic inflammation of the sinonasal mucosa and paranasal sinuses that has a substantial effect on quality of life and daily functioning of the patients. It is an increasing upper airway health problem that currently affects between 6 and 15% of the Caucasian population. (1, 2) CRS is clinically subdivided in CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). In the literature, CRSwNP is generally the more severe phenotype and is typically characterized by a type 2 "eosinophilic" inflammation, while CRSsNP is considered a type 1 "neutrophilic" inflammation. (3) While this traditional type 2 – type 1 classification in CRS is still valid in general, recent reports in asthma suggest that the role of neutrophils in type 2 airway inflammation might be far more important than initially thought. (4-6)

Therapies for CRSwNP nowadays mainly focus on targeting type 2 inflammation and considerable progress has been made in endotyping and treatment. (7-10) Typically, CRSwNP patients frequently suffer from recurrence after surgery or need repeated oral corticosteroid courses with potential long-term risks. (11) Non-responsiveness to oral corticosteroids may partially be caused by a co-existing neutrophilic inflammation, as described in both asthma and CRSwNP. (12, 13)

In asthma, the presentation of a mixed eosinophilic-neutrophilic inflammation, associated with a mixed type 2 – type 17 inflammation, is linked to a more severe and harder to control phenotype. (4) A recent cluster analysis from our group showed that clusters with the most severe clinical phenotype were characterized by a severe type 2 inflammation, but also by elevated levels of neutrophilic marker proteins. (14) On the other hand, the cluster analysis showed a group of CRSwNP patients with only neutrophil markers elevated. (14) During neutrophil and eosinophil extracellular trap cell death (NETosis and EETosis), cytosol and granule proteins are mixed with condensed DNA and released in the extracellular space, where they can affect the local inflammation. (15) Recent studies described EETosis in CRSwNP tissue, associated with Charcot Leyden Crystal (CLC) deposition; CLCs were found responsible for the induction and maintenance of neutrophilic inflammation in

CRSwNP. (15-18) We therefore sought to further characterize the driving forces of neutrophilic inflammation and its relationship to eosinophilic inflammation in severe type 2 immune reactions.

2. Methods

2.1. Sample collection:

Tissue and blood samples were collected from patients of the Department of Otorhinolaryngology at Ghent University Hospital (Belgium) after receiving written informed consent for inclusion. Nasal polyps and/or sinus mucosal tissue samples from patients undergoing functional endoscopic sinus surgery (FESS) for CRSwNP (n=56) or CRSsNP (n=35) and inferior turbinate (IT) from healthy patients (undergoing surgery for anatomical obstruction) (n=27) were collected at the Ghent University Hospital between February 2012 and June 2018. Tissue samples were either snap-frozen or fixed in paraformaldehyde and embedded in paraffin. For the isolation of neutrophils from peripheral blood, 100 ml blood was obtained from healthy volunteers and processed within 15 minutes after collection. None of the patients took oral- or intranasal glucocorticoids, nor antibiotics within 4 weeks before surgery. Clinical data and symptom surveys were collected from all patients that were enrolled in the study (Table 1). The study was approved by the local Ethics Committee of Ghent University Hospital.

2.2. Staining

2.2.1. Immunohistochemistry (IHC)

To prepare tissue slides for IHC and IF, nasal polyp and control tissues were fixed in 4% paraformaldehyde and embedded in paraffin. 4 µm thick slides were first deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Slides were blocked with 7.5% BSA in PBS and incubated with primary antibodies (monoclonal mouse anti-human MBP, BMK13, Monosan; monoclonal mouse anti-human NE, NP57, Dako; polyclonal rabbit anti-human histone H3, Abcam) for 16h at 4°C. The primary antibodies were visualized via an alkaline phosphatase linked secondary antibody kit according to manufacturer's instructions (Dako). Finally, the slides were counterstained with hematoxylin for 1 min and mounted with Aquatex™ mounting medium (Merck). Isotype controls

were included for all samples. Total number of positive cells were counted and expressed as number of cells/mm^2 tissue.

2.2.2. Immunofluorescence (IF)

Paraffin slides were prepared and deparaffinized as mentioned before. After antigen retrieval (EET-, CitH3- and Gal10-staining), slides were blocked with 7.5% BSA in PBS and incubated with primary antibodies (monoclonal mouse anti-human MBP, BMK13, Monosan; monoclonal mouse anti-human NE, NP57, Dako; polyclonal rabbit anti-human histone H3, Abcam; monoclonal mouse anti-human galectin-10, 561603, R&D) for 16h at 4°C. The primary antibodies were visualized via FITC-conjugated polyclonal goat anti-mouse or anti-rabbit IgG secondary antibody (Thermofisher). Slides were mounted with DAPI-containing mounting medium for nuclear counterstain. The slides were analyzed with a confocal laser-scanning microscope (Leica MicroSystems). For each patient, NETs, EETs and CLCs were counted in 10 random selected fields throughout the tissue. The number of cells involved in NET or EET formation were counted and normalized to the number of neutrophils or eosinophils respectively, and expressed as % of NET- or EET-generating neutrophils or eosinophils.

2.3. Cytokine and protein measurements

Snap frozen tissues were weighed, homogenized and centrifuged as described previously. (19) The samples were assayed for IL-5, IL-6, IL-8, IL-17, MPO and TNF-α using commercially available Luminex kits from R&D, and IFNγ using a commercially available Quantikine ELISA from R&D Systems (Minneapolis, Minnesota, USA). Levels were measured on a Bio-Plex 200 Array Reader (Bio-Rad, Hercules, CA, USA). Eosinophilic cationic protein (ECP), IgE and *Staphylococcus aureus* enterotoxin specific IgE (SE-IgE, staphylococcal enterotoxin A, staphylococcal enterotoxin C and toxic shock syndrome toxin-1) were measured using the UniCAP method (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Concentrations below detection limit were considered negative and were given the value half of the detection limit.

2.4. Quantitative PCR

RNA was extracted from snap frozen tissue samples as described previously. (15) In summary, RNA was isolated with the RNeasy Mini Kit (QIAGEN), subsequently cDNA was synthesized using the iScript Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was used to quantify mRNA levels of Gal10. The calibrated normalized relative quantities (CNRQs) were calculated with the qBase+ software (Biogazelle, Belgium). The primers were commercially purchased from Bio-Rad.

2.5. Flow cytometry analysis

The fraction of activated neutrophils in tissue of CRSsNP and CRSwNP patients was analyzed via flow cytometry, based on reduced expression of CD62L. Single cell suspension was prepared as described before. (20) Cells were labeled with near-IR life-dead dye (Invitrogen), PE-Cy7 anti-CD45 (BioLegend), PerCP-Cy5.5 anti-CD11b (BioLegend), Pacific blue anti-CD14 (BioLegend), PE anti-CD15 (BioLegend), FITC anti-CD16 (BD Pharmingen) and APC anti-CD62L (BioLegend). Neutrophils were gated by subsequent gating for viable cells (life-dead staining), single cells (FSC-A/FSC-H), leukocytes ($CD45^+$), myeloid cells ($CD11b^+$), granulocytes ($CD14^{-/dim}$) and neutrophils ($CD16^+/CD15^+$), based on FMO's, included for each marker. Activated neutrophils are expressed as % $CD62L^-$ neutrophils.

2.6. Neutrophil protease activity assays

Neutrophil elastase and cathepsin G activity were analyzed in tissues of controls, CRSsNP and CRSwNP patients. Homogenates were obtained from frozen tissue as described before (19) and dissolved in PBS + P/S. Fluorometric neutrophil elastase activity assay kit (Sigma-Aldrich) and colorimetric cathepsin G activity assay kit (Abcam) were used as instructed by manufacturers. Duplicates and background controls were included for all samples in both assays.

2.7. Boyden chamber assay

Peripheral blood neutrophils were collected from whole blood of healthy volunteers as described before. (16) Tissue homogenates of controls, CRSsNP and CRSwNP patients were obtained as described above and dissolved in RPMI + P/S. Lower compartments were filled with RPMI + 2% FCS + P/S (TCM) as negative control or 100 µg/ml tissue homogenate of healthy controls, CRSsNP or

CRSwNP. Subsequently, for each condition, 10^5 neutrophils were primed for 20 minutes with 100 ng/ml GM-CSF (PeproTech) and then allowed to migrate to the lower compartment through 5 µm pore size filters (VWR International) for 90 minutes at 37°C. The migrated neutrophils were collected, stained with a May Grunwald Giemsa stain and quantified for each condition. Two independent experiments were performed and results were expressed as migration index compared to TCM.

2.8. Neutrophil survival assay

Peripheral blood neutrophils were isolated and tissue homogenates were prepared as described before. 10^6 neutrophils were incubated in 100 µg/ml tissue homogenate of healthy controls, CRSsNP or CRSwNP for 8h at 37°C. After incubation, apoptotic neutrophils were stained using an Annexin V apoptosis detection kit (BD Pharmingen, Belgium) and analyzed using FACS Canto II with FACS Diva software (BD Bioscience).

2.9. Statistical analysis

Statistical analysis was performed using the Prism Graphpad version 8 software program. Mann-Whitney U test was used to evaluate statistical differences between two groups. Kruskal-Wallis test followed by a multiple comparison test was used to evaluate statistical differences between multiple groups. Correlations were analyzed with a non-parametric Spearman correlation test. Significances were expressed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < .0001$. P-values less than or equal to 0.05 were considered as statistically significant.

3. Results

3.1. Severe neutrophilic infiltration and inflammation in CRSwNP vs. CRSsNP.

In line with previous reports, we found that CRSwNP is clearly associated with type 2 inflammation, while CRSsNP is generally more related with type 1 inflammation. Tissue levels of IL-5 were significantly increased in CRSwNP compared to CRSsNP ($p < .0001$) and healthy controls ($p < .001$) (Fig. E1A), and tissue levels of IFN-γ were significantly ($p < .05$) increased in CRSsNP compared to CRSwNP and healthy controls (Fig. E1B). The characterization of the studied patients is presented in Table 1.

However, characterization of the tissue in terms of eosinophilic or neutrophilic infiltration showed a more versatile image than anticipated based on cytokine profiles in both CRSwNP and CRSsNP patients. Both patient groups contained cases with low granulocyte infiltration, predominantly neutrophilic, predominantly eosinophilic and mixed neutrophilic-eosinophilic infiltration (Fig. 1A). Interestingly, most of the patients with severe type 2 CRSwNP had a mixed neutrophilic-eosinophilic inflammation.

Based on these observations in the tissue, we quantified the number of eosinophils and neutrophils in both patient groups. Numbers of eosinophils were significantly elevated in CRSwNP compared to CRSsNP ($p < .0001$) and healthy controls ($p < .001$) (Fig. E2A). In line with this, we found that tissue ECP levels were significantly increased in CRSwNP compared to CRSsNP ($p < .01$) and healthy controls ($p < .0001$) (Fig. E2B). Numbers of neutrophils were significantly elevated in CRSwNP compared to CRSsNP ($p < .05$) and healthy controls ($p < .01$) (Fig. 1B). In line with this, we found tissue levels of MPO was significantly ($p < .01$) elevated in CRSwNP compared to controls (Fig. 1C), and IL-8 and IL-6 were significantly increased in CRSwNP compared to CRSsNP (resp. $p < .05$ and $p < .01$) and healthy controls ($p < .01$ and $p < .05$) (Fig. 1D-E). These findings showed a profound neutrophilic inflammation, co-existing with eosinophilic inflammation in severe type 2 CRSwNP patients.

Interestingly, no intergroup differences were observed in tissue levels of IL-17, a major driving force for the recruitment, survival and activation of neutrophils (Fig. 1F). To investigate the possible involvement of a type 17 inflammation, IL-17 levels were related to the number of neutrophils in the tissue. Interestingly, while the number of neutrophils was significantly ($p < .05$) elevated in the CRSsNP patients with high IL-17 levels compared to the group with low IL-17, we found no significant increase in ratio of neutrophils in CRSwNP patients with high IL-17 compared to the CRSwNP patients with low IL-17 levels (Fig. 1G).

3.2. Neutrophil infiltration associated with type 2 inflammation in CRSwNP

As recently reported, the presence of CLCs – the crystalized form of galectin-10 (Gal10) protein – results from the process of EETosis and is a hallmark of type 2 inflammation in airway immunity. (17, 18) Therefore, Gal10 mRNA expression levels were measured and found significantly upregulated in CRSwNP patients compared to healthy controls ($p < .0001$) and CRSsNP ($p < .05$). In addition, Gal 10 mRNA expression was also significantly ($p < .01$) upregulated in CRSsNP compared to healthy controls (Fig. 2A). No significant influence of comorbid asthma or allergy on Gal10 mRNA levels was found in the patient groups (Data not shown). The numbers of CLCs were quantified and found significantly ($p < .01$) upregulated in CRSwNP compared to healthy controls (Fig. 2B). Interestingly, the number of neutrophils correlated positively with the relative fractions of EETs ($p < .001$; $r = .8047$; Fig. 2C) and the numbers of CLCs ($p < .01$; $r = .6504$; Fig. 2D) in type 2 CRSwNP, but not in CRSsNP and controls.

3.3. Increased neutrophil activation in type 2 CRSwNP

Since both neutrophils and its activating cytokines are increased in CRSwNP, we analyzed and compared neutrophil activity in both CRSsNP and CRSwNP tissue. Neutrophils are known to shed of CD62L upon activation. Therefore, we studied the neutrophil activity directly by analyzing tissue derived neutrophils on reduced CD62L expression via FACS. The percentages of activated ($CD62L^-$) neutrophils were significantly ($p < .05$) increased in tissue of type 2 CRSwNP compared to CRSsNP patients (Fig. 3A). In addition, the proteolytic activity of both cathepsin G was significantly ($p < .01$)

increased in the tissue of CRSwNP compared to controls, and the activity of neutrophil elastase was significantly ($p < .05$) increased in CRSwNP compared to controls and CRSsNP (Fig. 3C-D).

3.4. Neutrophils more prone to go into NETosis in CRSsNP tissue.

Similar to eosinophils, neutrophils have the ability to generate extracellular traps in a process called NETosis. We quantified NETs in the tissue and found that NETosis was present in 67% of all CRSsNP patients and 64% of the CRSwNP patients. The rate of NETosis was significantly ($p < .05$) increased in CRSsNP compared to healthy controls (Fig. 4A). Tissues were also stained for CitH3, an early marker of NETosis, and the presence of $CitH3^+$ neutrophils was quantified and found significantly ($p < .01$) increased in CRSsNP compared controls (Fig. 4B). Elastase was stained positive on the same spots as CitH3, which confirmed the specificity of the CitH3 staining in neutrophils.

In the tissue of CRSwNP, NETosis was mainly found at the edges of (denuded) epithelium (Fig. 4C) and was colocalized with signs of bacterial colonization (Fig. 4D). In CRSsNP, NETosis was mainly found in the stroma and underneath a clear thickened basement membrane associated with denuded epithelium (Fig. 4E).

3.5. Decreased neutrophil survival in CRSwNP cytokine environment

Differential neutrophil migration among the different endotypical environments were studied via Boyden-chamber assays; but did not reveal significant differences in neutrophil migration between the different patient groups (Fig. 5A). To study the influence of the cytokine environment on neutrophil survival, peripheral blood neutrophils were cultured in tissue homogenates of the different patient groups and the rate of apoptosis was analyzed after 24h of incubation via FACS analysis. Neutrophils cultured in CRSsNP homogenates showed significantly ($p < .05$) lower rates of apoptosis compared to neutrophils cultured in homogenates of healthy controls and CRSwNP (Fig. 5B).

To investigate the possible involvement of IL-17 in neutrophil survival, the percentages of apoptotic neutrophils after incubation with homogenates were related to levels of IL-17 in these homogenates. Interestingly, no correlation was found in CRSwNP homogenates, while neutrophil apoptosis was significantly ($p < .05$; $r = 0.5912$) inversely correlated with levels of IL-17 in the CRSsNP homogenates (Fig 5C-D).

4. Discussion

While we confirmed the general consideration that CRSsNP is mainly mediated by non-type 2 and CRSwNP by a type 2 inflammatory pattern, we here demonstrate a very heterogeneous image of neutrophilic and eosinophilic inflammation among all CRS patients. (3, 14, 21) Both neutrophil tissue counts and neutrophil-related protein levels demonstrated an increased neutrophilic inflammation in the CRSwNP patient group. This is in line with a previous cluster analysis showing a markedly increased presence of neutrophilic marker proteins in the high type 2 clusters containing the most severe and difficult to treat CRSwNP patients. (14) Some reports claim that the number of neutrophils is elevated in CRSsNP compared to CRSwNP, while other studies find that the numbers are comparable or elevated in CRSwNP. (22-25) These contradicting results are more than likely attributed to the existence of different CRS endotypes of which some have a clear neutrophilic inflammation co-existing with eosinophilia.

Interestingly, neutrophils are also more activated and neutrophilic proteases elastase and cathepsin G have increased activity in type 2 CRSwNP. This indicates that neutrophils are not only more frequent in a type 2 environment, but they also have a higher potential to affect local inflammation via increased proteolytic activity. Elastase and Cathepsin G are known to enhance secretion and activation of IL-1 family cytokines as IL-1β and IL-33. (26) These cytokines are key players in the induction of type 2 responses as they function as chemoattractant for Th2 cells and stimulate the production of type 2 cytokines in eosinophilic nasal polyps. (27-29) In addition, neutrophil elastase itself has a major impact on airway inflammation as it enhances goblet cell hyperplasia and mucus production. (30)

Despite the elevated number of neutrophils and increased neutrophil activity in CRSwNP patients, no elevated neutrophil migration nor survival in CRSwNP tissue homogenates could be found *in vitro*. We speculate that neutrophil clearance is affected in CRSwNP tissue or that the active recruitment of neutrophils into CRSwNP tissue requires intact tissue. In severe asthma, a mixed neutrophilic-

eosinophilic inflammation is associated with a mixed type 2/17 inflammation. Despite of the observed similarities in mixed airway inflammation, no remarkable elevation of IL-17 was observed in CRSwNP patients, nor was there any correlation between IL-17 levels and number of neutrophils in these patients. This is in line with a recent cluster analysis by P. Tomassen et al. showing increased levels of neutrophil related proteins, but no elevated levels of IL-17 in high type 2 CRSwNP patients. (14) We recently showed that CLCs, prominently present in severe CRSwNP patients, can evoke a neutrophilic inflammation. (16) In addition, increased neutrophilic influx was observed in murine lungs upon injection with CLCs. (17) In line with these findings we showed here that the number of neutrophils in the tissue was correlated with the extend of EETosis and CLC deposition in CRSwNP. Despite the presence of CLCs in CRSsNP patients, however, no correlation with neutrophil infiltration was found. (31) This could possibly be attributed to the differences in CLC-size we observed in both groups, since size of extracellular pathogens are also known to affect mechanism of clearance by neutrophils. (32) *S. aureus* colonization – another hallmark of CRSwNP – is also linked to increased neutrophil migration in CRSwNP and could therefore have a prominent role *in vivo* triggering neutrophilia in CRSwNP. (33-35) Since the general bacterial load does not differ between CRSsNP and CRSwNP, we believe that the increased neutrophil inflammation observed in CRSwNP is not only caused by bacterial colonization. (36) However, variations in the composition of sinus bacterial microbiota itself was previously shown to be linked to CRS heterogeneity and may therefore also contribute to differences in neutrophilic activation and NETosis. (36, 37) In this study, differences in local bacterial composition in relation to neutrophilic inflammation were not analyzed, but would be interesting for future studies.

A considerable fraction of neutrophils was found to undergo NETosis both in the CRSsNP and CRSwNP patient groups. Both relative and absolute numbers of neutrophils undergoing NETosis were increased in CRSsNP. This was in line with our finding of increased neutrophil survival in CRSsNP. NETosis has been proposed as a type of slow programmed cell death that is prone to occur in neutrophils with increased survival. (38) While CitH3 is an early and consistent marker of NETosis,

NETs themselves can be quite fragile. Extracellular traps can be disintegrated in a relative fast manner, especially in a severe type 2 environment with increased numbers of macrophages and bacterial DNases. (39-41) Multiple stimulants can induce NET-formation and even the type of NETosis – vital or suicidal – is dependent on its environmental incentives. (42) It has recently been reported that CLCs could induce NETosis and neutrophilic inflammation in CRSwNP, and that NETosis could induce a type 2 response via release of extracellular dsDNA. (5, 16) In CRSwNP, NETosis was mainly located at sites of sometimes denuded epithelium. Interestingly, neutrophilic mediators as cathepsin G and elastase are extensively released during NETosis and stimulate type 2 immune responses as described above. (26, 43)

While NETosis in CRSwNP was mainly found subepithelial and closely collocated with signs of bacterial infection, NETosis in CRSsNP was mainly found deeper in the tissue. It remains to be clarified if the NETosis is activated via similar pathways in the different endotypes. NETosis in CRSwNP may be driven by type 2 related mediators or may be related to the increased bacterial colonization and biofilm formation at sites of denuded epithelium. In CRSsNP, NETosis is mainly found in the stroma and underneath a clearly thickened membrane, without a clear association with bacterial colonization. This might indicate that other mediators such as IFN-γ or IL-12, both known inducers of NETosis and specifically elevated in those patients, might initiate the NETosis in the CRSsNP tissue. (44, 45)

Due to predominant Th2 inflammatory pattern, therapies treating chronic airway diseases nowadays – as anti-IL5(Ra), anti-IL4R/IL13R or glucocorticoids – mainly focus on targeting the eosinophilic Th2 inflammation. (7-10, 13) With the establishment of these therapies considerable advances have been made, but still a part of the patients does not respond to the treatment. (11, 46, 47) We here showed that the contribution of neutrophils – by themselves via increased activity, and via increased influx and NETosis or in combination with eosinophilic inflammation – could be far more important than initially thought, especially in a Th2 context. Interestingly, as CLCs are highly stable and remain

present at sites of inflammation for months, they could still affect neutrophilic inflammation long after the start of treatment. (16, 17) In addition, recent studies showed reduced responsiveness to corticosteroids in neutrophilic asthma and CRSwNP. (12, 13) All this indicates that neutrophilic inflammation is currently overlooked in assignments of therapies targeting the eosinophilic Th2 inflammation and may need more attention to enable further improvement of treatments of the patients. Therefore, we speculate that the identification of increased neutrophil activation markers in CRS patients may evolve as one of the critical parameters in determining treatment response in CRS patients. The development and co-administration of novel drugs targeting CLC-activated neutrophils may complete the response to the Type 2 cytokine antagonists in patients with a mixed eosinophilic-neutrophilic inflammation.

Overall, the behavior of neutrophils observed in CRSwNP is comparable to neutrophilic behavior in infections or other chronic type 2 mediated airway diseases. At sites of infection, neutrophils also induce NET-formation and secrete proteases to promote bacterial clearance. (48) In asthma, neutrophils display a 5 days longer lifespan through decreased apoptosis. (30) This increased life span may set the basis for increased activation and development of NETosis, which could contribute to the severity of the disease. Also in nasal turbinate samples of patients with allergic rhinitis the percentage of activated neutrophils was significantly higher than non-activated neutrophils, while this was not the case in the tissue of non-allergic patients. (49) The difference between regular infections and infections in chronic type 2 diseases seems to be the triggers of the local neutrophilic inflammation. Migration of neutrophil to sites of infections are mainly mediated by DAMPs, CXCL-8 and leukotrienes, while in type 2 inflammations, this traditional way of neutrophilic migration seems to be overruled by mediators of eosinophilic type 2 inflammation such as CLCs. (16-17, 48) Also at sites of infection, neutrophils release proteins and form NETs in direct response to bacterial invasion, while in CRSwNP these functions are not exclusively triggered by bacterial infection, but also by type 2 mediators such as eosinophils and CLCs.

In conclusion, we here demonstrated a profound involvement of neutrophils in severe type 2 CRSwNP, induced by products of eosinophilic inflammation, and independent of IL-17. Neutrophils have a high potential to affect local inflammation in CRSwNP through an increased activation status, increased proteolytic activity of elastase and cathepsin G, the formation of NETs and finally to aggravate the type 2 inflammation. These mechanisms could result in a circle of aggravation of inflammation in the most severe CRSwNP patients with mixed eosinophilic-neutrophilic inflammation, leading to the insensitivity to GSCs and type 2 biologics; this hypothesis needs to be further supported.

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6. Legends of Tables

Table 1: Patient characterization

Chronic rhinosinusitis with (CRSwNP) and without (CRSsNP) nasal polyps, Female (F), Male (M), present (+), not present (-), standard deviation (SD), immunoglobulin E (IgE), *S. aureus* enterotoxin specific immunoglobulin E (SE-IgE), interleukin-5 (IL-5), eosinophilic cationic protein (ECP), interleukin-17 (IL-17), tumor necrosis factor alpha (TNF-α), myeloperoxidase (MPO). Allergy was defined as 'present' when the patient had a positive skin prick test for at least one of the inhalant allergens commonly tested in our region.

7. Legends of Figures + Supplemental Figures

Figure 1: Differential neutrophilic and eosinophilic infiltration in CRSsNP and CRSwNP, with increased neutrophilic inflammation in CRSwNP (A) Inflammatory subgroups of CRSwNP and CRSsNP based on the mean neutrophil counts (resp. 65 and 28 cells/mm²) and the mean eosinophil counts (resp. 811 and 16 cells/mm²). (B) Quantification of neutrophils per mm² in tissue of healthy controls, CRSsNP and CRSwNP patients. (C-F) MPO, IL-8, IL-6 and IL-17 protein levels in the tissue of healthy controls, CRSsNP and CRSwNP patients, measured via UniCAP and ELISA. (G) Ratio of neutrophils in tissue of CRSsNP and CRSwNP patients with high and low protein levels of IL-17, based on the median tissue concentration of IL-17 in both groups (resp. 27.28 and 15.02 pg/ml). *Levels of statistical significance are expressed as *p < .05, **p < .01, ***p < .001 and ****p < .0001.*

Figure 2: Elevated CLC deposition in CRSwNP associated with neutrophilic infiltration (A) Relative galectin-10 gene expression in the tissue of healthy controls, CRSsNP and CRSwNP patients, expressed as calibrated normalized relative quantity (CNRQ) values. (B) Quantification of Charcot-Leyden crystals per high power field (hpf) in the tissue of healthy controls, CRSsNP and CRSwNP patients. Scatter plots of numbers of neutrophils per mm² with (C) ratios of EETs and (D) numbers of

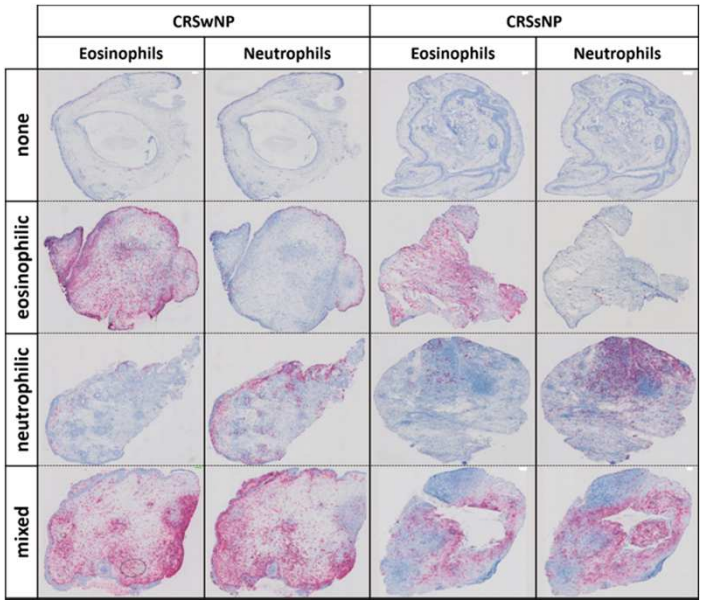
CLCs per mm² in tissue of CRSwNP patients, showing significant correlations ($p < .001$; $r = .8047$ and $p < .01$; $r = .6504$), determined via Spearman correlation. *Levels of statistical significance are expressed as *p < .05, **p < .01, ***p < .001 and ****p < .0001.*

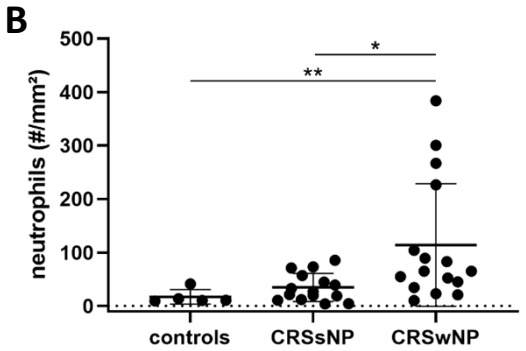
Figure 3: Increased neutrophilic activation in CRSwNP. (A) FACS analysis showed significantly ($p < .05$) increased ratios of activated ($CD62L^-$) neutrophils in mucosal tissue of CRSwNP compared to CRSsNP patients. Significantly increased activity of cathepsin G ($p < .01$) and neutrophil elastase ($p < .05$) was observed in mucosal tissue of CRSwNP compared to controls and CRSsNP patients. *Levels of statistical significance are expressed as *p < .05, **p < .01, ***p < .001 and ****p < .0001.*

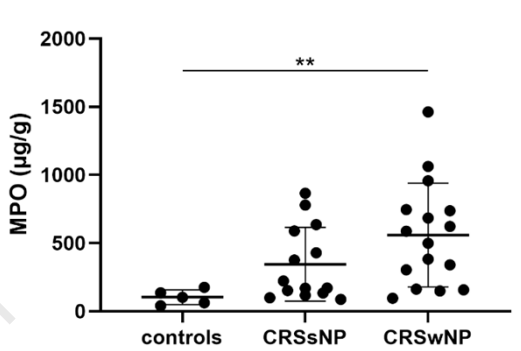
Figure 4: Increased NETosis in CRSsNP. (A) Quantification of NETs in the tissue of healthy controls, CRSsNP and CRSwNP patients, expressed as % of neutrophils generating NETs. (B) Quantification of CitH3-positive neutrophils in the tissue of healthy controls, CRSsNP and CRSwNP patients, based on IHC staining. (C-E) Immunofluorescent staining of CitH3 and elastase, showing the presence of NETosis in CRSwNP and CRSsNP. (C) NETosis at the edges of denuded epithelium in CRSwNP, (D) colocalized with signs of bacterial colonization (arrowhead). (E) NETosis underneath a clear thickened basement membrane in CRSsNP, white line represents the putative epithelial layer. *Levels of statistical significance are expressed as *p < .05.*

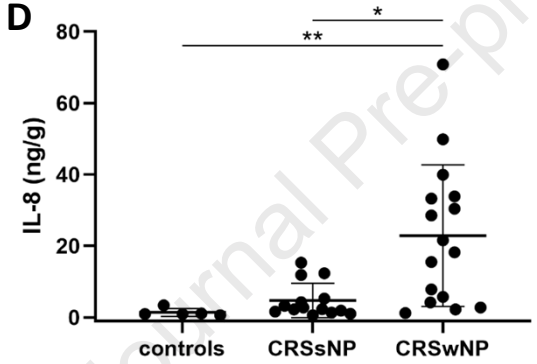
Figure 5: Decreased neutrophil apoptosis in CRSsNP. (A) Quantification of neutrophils migrated to tissue homogenates of healthy controls, CRSsNP and CRSwNP patients. (B) Ratios of apoptotic neutrophils after 24h culturing in tissue homogenates of healthy controls, CRSsNP and CRSwNP patients. (C-D) Scatter plots of neutrophil apoptosis (%) and levels of IL-17 in homogenates, showing a significant ($p < .05$; $r = -.5912$) inversely correlation in CRSsNP (C) and no correlation in CRSwNP (D), determined via Spearman correlation. *Levels of statistical significance are expressed as *p < .05 and **p < .01.*

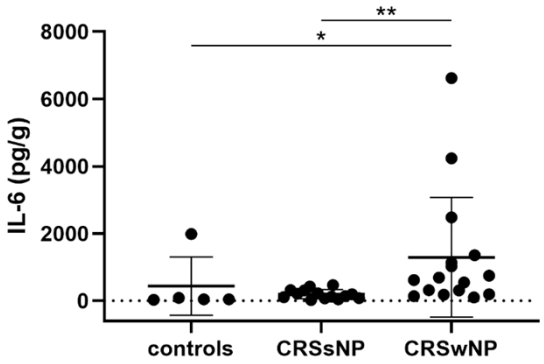
		Controls	CRSsNP	CRSwNP
Total cases	(#)	27	35	56
Gender	F/M	7/20	12/23	19/37
Age	median/range	30 (20 - 58)	44 (18 - 67)	46 (19 - 92)
Ethnicity		Caucasian	Caucasian	Caucasian
Previous surgeries	(yes/no)	(3/24)	(12/23)	(33/23)
	Range # surgeries	0-1	0-3	0-4
Allergy	(+/-)	12/14	18/17	33/22
	missing cases (#)	1	-	1
Asthma	(+/-)	4/23	4/31	28/27
	missing cases (#)	-	-	1
Tissue concentrations (Mean ± SD)				
IgE	U/g	66.56 ± 76.81	578.46 ± 1688.78	932.26 ± 1329.99
SE-IgE	UA/g	1.07 ± 2.72	0.18 ± 2.27	4.16 + 5.43
IL-5	pg/g	4.84 ± 10.21	89.72 ± 122.83	537.32 + 680.13
ECP	µg/g	0.46 ± 0,67	5.67 ± 5.41	41.29 + 123.88
IL-17	pg/g	52.27 ± 81.37	55.83 ± 89.83	42.59 + 89.58
TNF-α	pg/g	7.99 ± 15.51	18.65 ± 23.94	22.99 + 23.88
MPO	µg/g	103.43 ± 54.16	344.85 ± 270.22	559.48 + 379.88

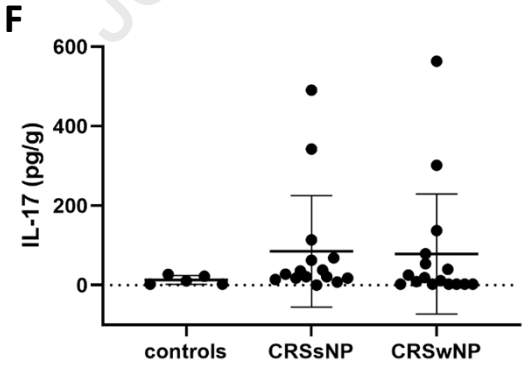


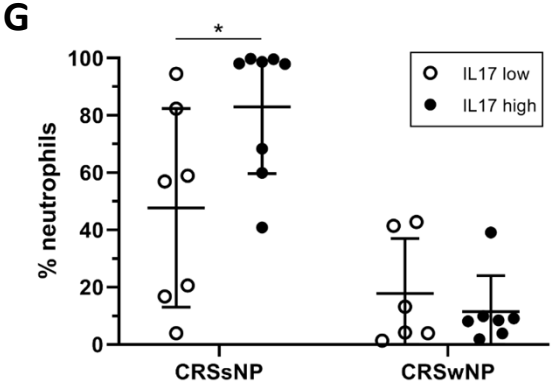


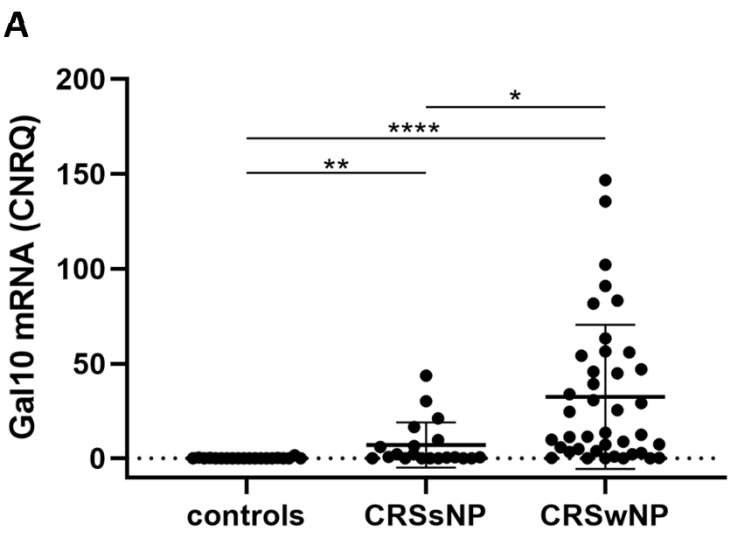


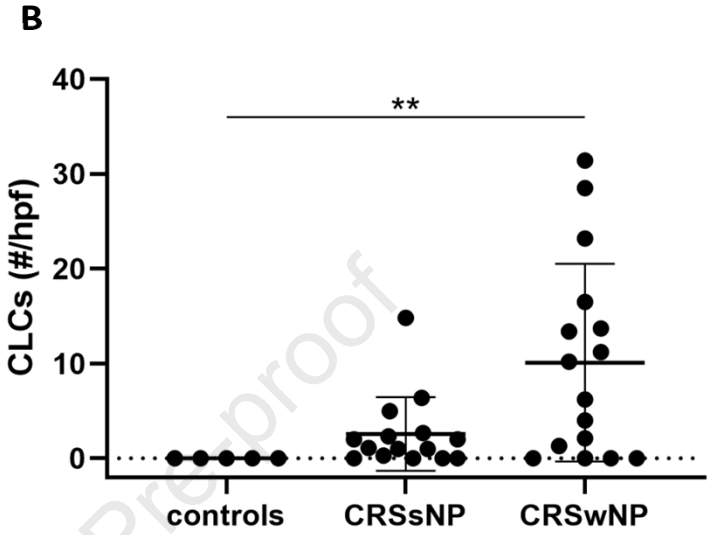


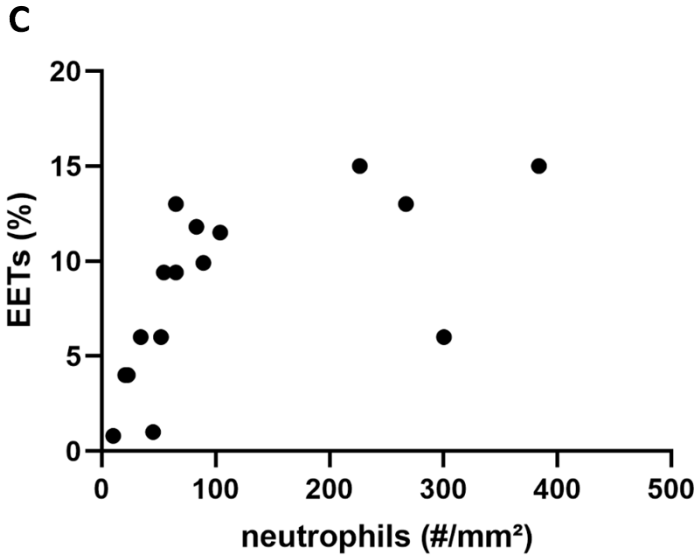


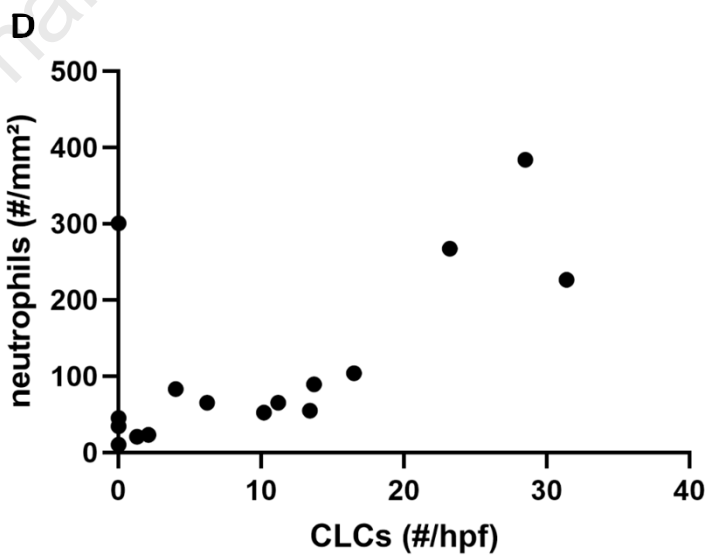


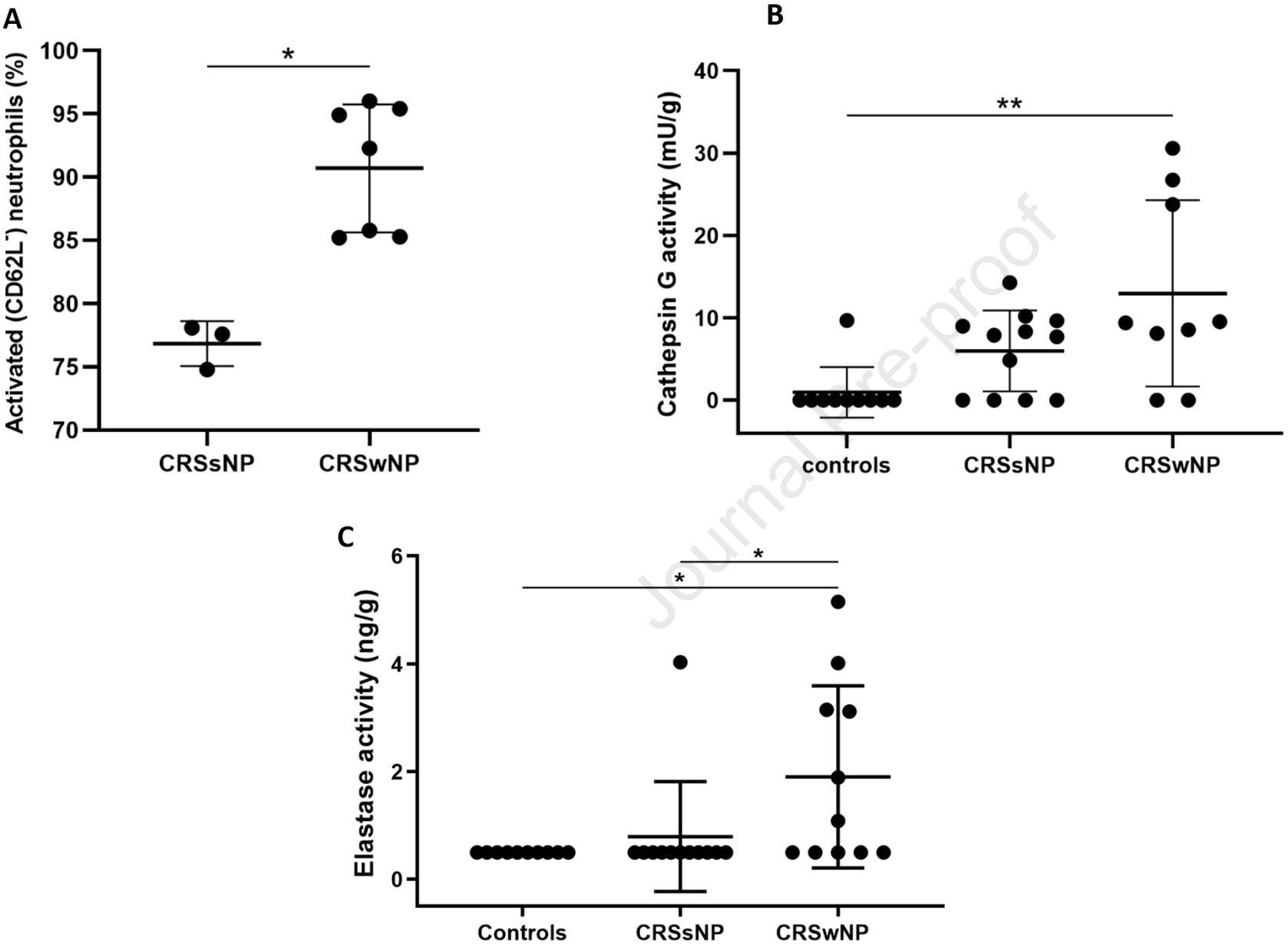


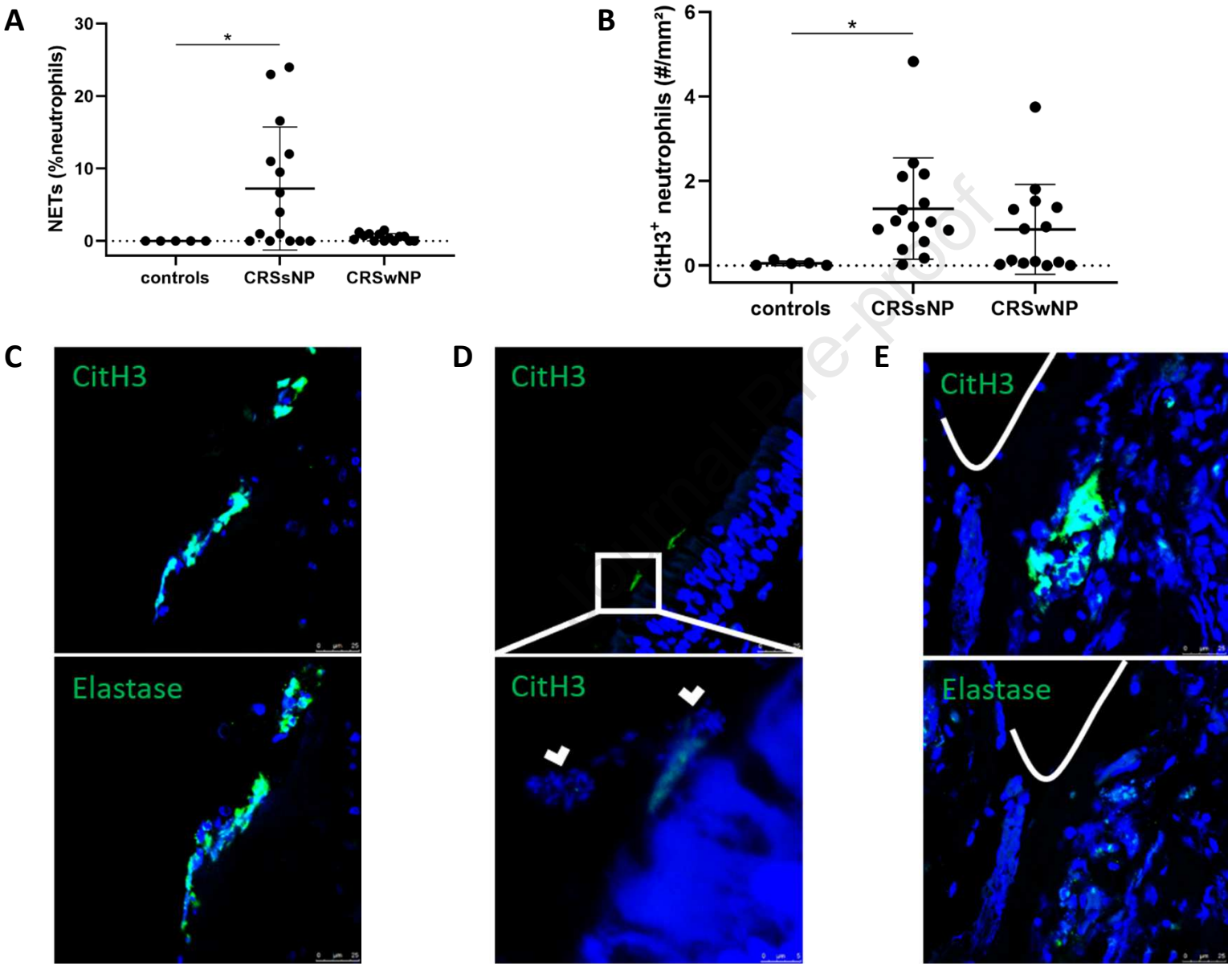


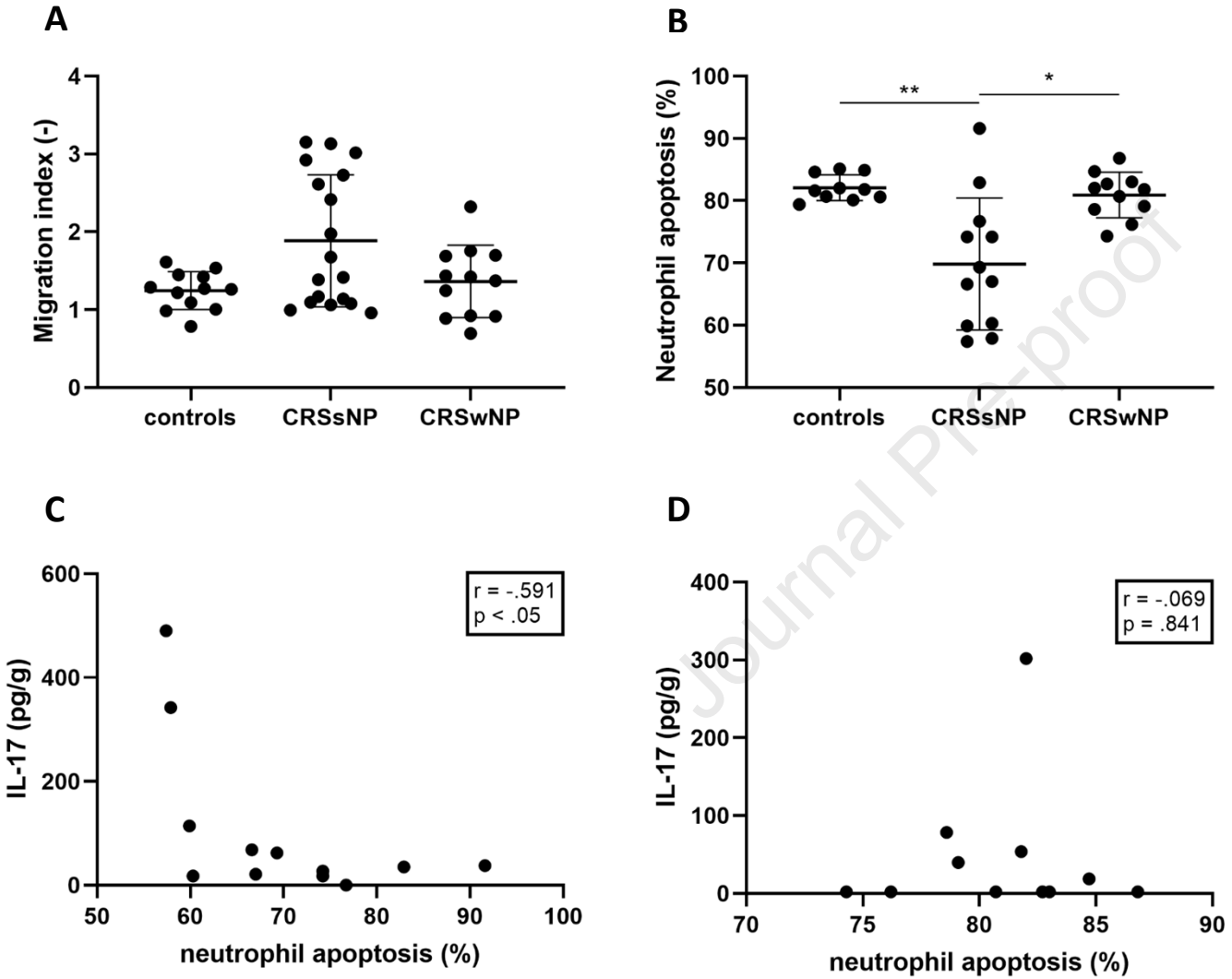












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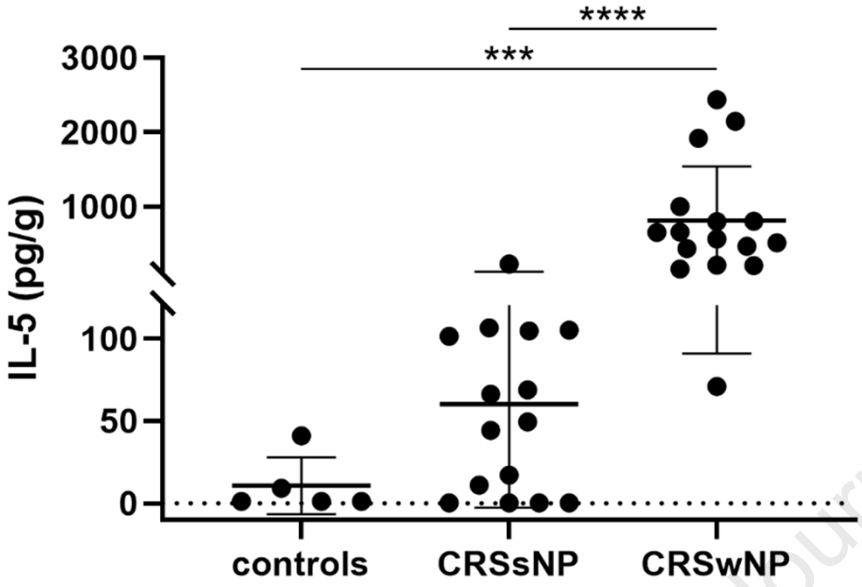

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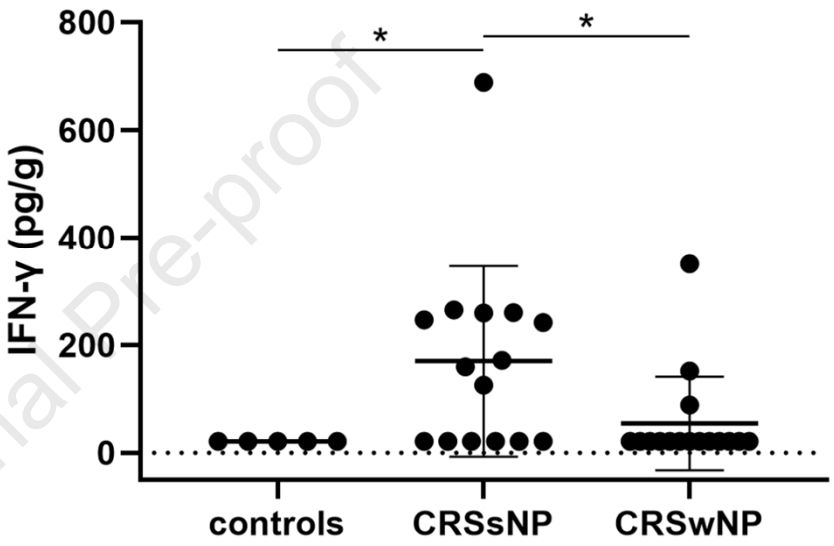

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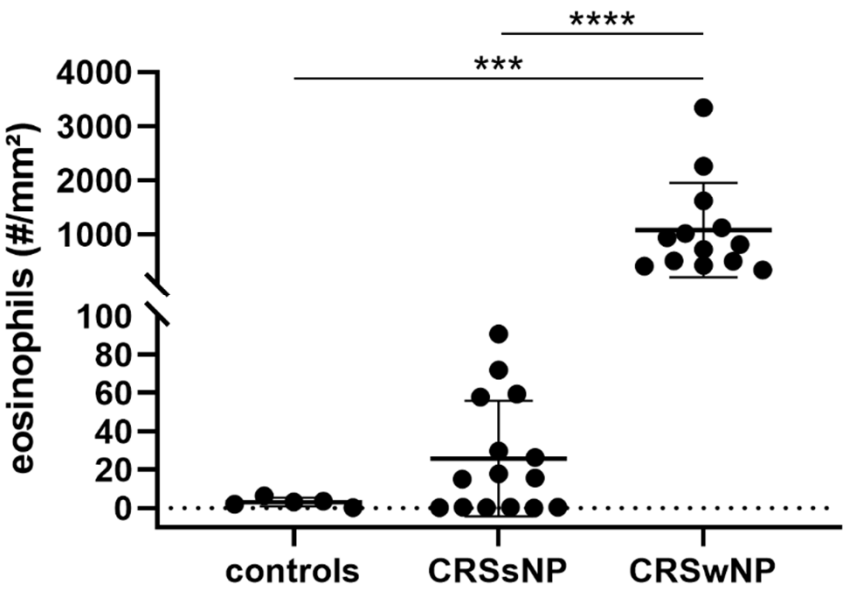

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