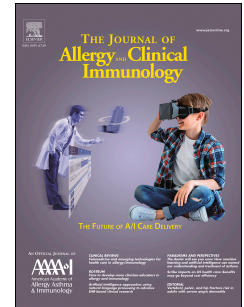


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Studies of the Role of Basophils in Aspirin Exacerbated Respiratory Disease Pathogenesis

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Conflicts of Interest:

RC, DC, KH, JH, JN, JP, AS, SS, LS, and KW reports no conflicts of interest. WS served on an advisory board for GlaxoSmithKline. AK reports personal fees from Astellas Pharmaceuticals and a gift for his research from Lyra Therapeutics. BT reports personal fees from Sanofi Regeneron/Genzyme, and OptiNose. AP reports personal fees from Sanofi Regeneron and personal fees and grants from AstraZeneca and Optinose. LG reports personal fees from Astellas Pharmaceuticals. RK reports personal fees from Genentech, GlaxoSmithKline, Sanofi, Novartis, Lyra Pharmaceutical, and Neurent. RS reports personal fees from Intersect ENT, Merck, GlaxoSmithKline, Sanofi, AstraZeneca/Medimmune, Genentech, Actobio Therapeutics, Lyra Therapeutics, Astellas Pharma Inc., and Otsuka Inc. RS also has Siglec-8 and Siglec-8 ligand related patents licensed to Allakos Inc.

Total Word Count: 3,795

This article has an online data supplement.

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The mean number of basophils (CD45⁺CD203c⁺FcεRI⁺CD117⁺) detected in AERD NP (147±28 cells/mg tissue) was significantly elevated compared to CRSwNP (69±20 cells/mg tissue; p=0.01). The number of circulating basophils was significantly elevated in AERD (p=0.04). Basophils in NP had significantly higher CD203c and CD63 MFI compared to blood in both conditions (p<0.01). Basophils from AERD NP had lower expression of the granule content marker 2D7 than matched blood (p<0.01) or NP of CRSwNP (p=0.06), suggesting ongoing degranulation. Basophil 2D7 MFI significantly correlated with pulmonary function (r=0.62, p=0.02) and inversely correlated with sinonasal inflammation (r=-0.56, p=0.004).

Conclusions:

Increased basophil numbers and extent of ongoing degranulation in NP of patients with AERD compared to CRSwNP may contribute to the exaggerated disease pathogenesis and severity unique to AERD.

Abstract Word Count: 250

Key Messages:

- Basophils are elevated in nasal polyp and peripheral blood of patients with AERD compared to patients with CRSwNP.
- Basophils are activated in nasal polyps of both CRSwNP and AERD patients; however, basophils appear to have degranulated to a greater extent in AERD.
- The extent of basophil degranulation significantly correlated with clinical markers of enhanced disease severity.

Capsule Summary:

Increased basophil numbers and extent of ongoing degranulation in nasal polyps of patients with AERD compared to CRSwNP may contribute to the exaggerated disease pathogenesis and severity unique to AERD.

Key Words:

Aspirin Exacerbated Respiratory Disease, AERD, basophil, 2D7, nasal polyp, CRSwNP, chronic sinusitis

Abbreviations:

Aspirin Exacerbated Respiratory Disease (AERD)
Chronic Rhinosinusitis with Nasal Polyps (CRSwNP)
Cyclooxygenase (COX)
Inferior Turbinate (IT)

- 102 Mean Fluorescence Intensity (MFI)
- 103 Nasal Polyp (NP)
- 104 Lund-Mackay (LM)

Introduction

Aspirin Exacerbated Respiratory Disease (AERD) is a chronic inflammatory condition of the upper and lower respiratory tracts. Clinically, AERD is comprised of the triad of chronic rhinosinusitis with nasal polyps (CRSwNP), asthma, and intolerance to medications that inhibit the cyclooxygenase-1 (COX-1) enzyme. AERD is estimated to occur in 8-16% of patients with CRSwNP and 15% of patients with severe asthma^{1,2}. On average, patients with AERD have more severe sinonasal disease, undergo significantly more sinus surgeries, and are more likely to require daily oral corticosteroids than patients with CRSwNP alone or with asthma but lacking sensitivity to aspirin-like drugs². The underlying cellular and molecular mechanisms contributing to the enhanced severity and unique phenotype of AERD are not fully understood.

In Western countries, nasal polyps (NP) are predominantly characterized by type 2 inflammation^{3,4}. Levels of the type 2 cytokines IL-5 and IL-13 are elevated in NP when compared to healthy controls^{5,6} although no significant difference was observed between AERD and CRSwNP². Cells commonly associated with type 2 inflammation, including type 2 CD4⁺ T cells and group 2 innate lymphoid cells, are elevated in NP in the West⁷⁻⁹. Eosinophils are also significantly elevated in NP compared to healthy controls and their associated granule proteins are even higher in NP of AERD patients compared to those with only CRSwNP^{2,5,6,10}. Elevations in the number of circulating eosinophils are clinically associated with an increased risk of disease recurrence in patients with CRSwNP and AERD¹¹. Mast cells are elevated and also suspected to play a role in NP pathogenesis, especially in AERD where increased production of

mast cell mediators including Prostaglandin D₂ (PGD₂) and cysteinyl leukotrienes have been demonstrated and implicated in reactions to COX-1 inhibitors¹²⁻¹⁶.

Basophils are potent granulocytes that have long been associated with type 2 inflammation. These cells can secrete a variety of pro-inflammatory mediators, including histamine, cysteinyl leukotrienes, IL-4 and IL-13, that can all contribute to the type 2 allergic inflammatory response^{17,18}. Clinical studies found that basophils were significantly elevated in postmortem lung sections of asthmatics who died in status asthmaticus compared to lung from asthmatics who died from non-asthmatic causes^{19,20}. Activation of blood basophils has been used as a biomarker of disease in food allergy, allergic rhinitis, and asthma.

Despite evidence that basophils are important contributors to type 2 respiratory diseases, there are few studies evaluating basophils in NP. In a previous study, our group used immunohistochemistry to detect 2D7⁺ basophils and found elevations of these cells in CRSwNP tissue, but paradoxically not in AERD¹⁰. That study had some weaknesses as it did not quantify these cells rigorously, it did not assess the level of basophil activation or allow for changes in 2D7 during activation, and it did not relate basophils or basophil activation to markers of disease severity. In the current study, we significantly advanced our previous work on basophils by utilizing a flow cytometry-based approach to characterize, quantify, and compare basophils in NP of patients with AERD and CRSwNP. We found that basophils were significantly elevated and activated in CRSwNP and in AERD NP. We also found that levels of a marker of basophil degranulation correlated with enhanced clinical disease severity.

Methods

Study Population

All subjects signed informed consent and were recruited from the Division of Allergy and Immunology or Department of Otolaryngology clinics within Northwestern Medicine. Additional information is provided in the online supplement with demographic information listed in Table 1. The Institutional Review Board of Northwestern University Feinberg School of Medicine approved this study.

Isolation of cells

Cells were isolated from NP and peripheral blood as previously described⁸ with additional information provided in the online supplement.

Flow cytometric analysis

Protocols and reagents used for flow cytometric analyses of NP and peripheral blood are provided in the online supplement. Representative gating strategies are shown for eosinophils (Supplemental Figure 1) and mast cells and basophils (Supplemental Figure 2).

Statistics

Data was analyzed using a Chi-squared test, Mann-Whitney test, one-way ANOVA Kruskal-Wallis test with Dunn's test for multiple comparisons, and Spearman rank as appropriate. A p-value <0.05 was considered significant with further details provided in the online supplement.

Results

Identification of type 2 inflammatory granulocytes in nasal polyps

Previous work has shown that cells staining with the basophil-specific marker 2D7 were present and elevated in NP (Supplemental Figure 3)¹⁰. The reliability of 2D7 is variable as it is found in the granules of basophils, and degranulation of the cells can theoretically diminish staining of activated cells. We therefore undertook a challenging study in which we utilized flow cytometry of dispersed tissue cells to identify and characterize basophils along with other inflammatory cells in NP of AERD or CRSwNP patients. The average number of CD45⁺ cells (leukocytes) was elevated in AERD NP (5,606±1,150 cells/mg tissue) compared to NP from CRSwNP (3,504±649 cells/mg tissue) but this difference did not reach statistical significance ($p=0.09$, Mann-Whitney test) (Figure 1A). For reference, inferior turbinate (IT) of control patients without chronic rhinosinusitis had significantly fewer leukocytes (1,733±182 cells/mg tissue) than the NP subgroups ($p<0.007$, Kruskal-Wallis test) (Figure 1A). These findings demonstrate that NP have an exaggerated cellular inflammatory response compared to healthy sinonasal tissue.

To further characterize the inflammatory milieu within NP, we next evaluated various type 2-associated inflammatory granulocytes using flow cytometry. Eosinophils were identified as CD45⁺Siglec-8⁺Fc γ RI⁻ cells, while mast cells were defined as CD45⁺CD117⁺Fc γ RI⁺ cells. The average number of eosinophils was increased in AERD NP (6,277±2,548 cells/mg tissue) compared to CRSwNP (4,161±1,221 cells/mg tissue) but the difference was not significant ($p=0.54$) (Figure 1B). Similarly, there was a non-significant trend ($p=0.15$) toward an increase in

the average number of NP mast cells in AERD (191 ± 46 cells/mg tissue) compared to CRSwNP (124 ± 36 cells/mg tissue) (Figure 1C). However, IT of control patients had significantly fewer mast cells (15 ± 9) than either polyp subgroup ($p < 0.001$, Kruskal-Wallis test) (Figure 1C).

Basophils, defined as $CD45^+CD203c^+Fc\epsilon RI^+CD117^-$ cells, were significantly elevated in NP of AERD (147 ± 28 cells/mg tissue) compared to CRSwNP (69 ± 20 cells/mg tissue; $p = 0.01$) patients (Figure 1D). Basophils also trended to represent a larger proportion of total NP leukocytes in AERD (3.2%) compared to CRSwNP (2.2%; $p = 0.09$) (data not shown). For reference, IT of control patients without CRS had significantly fewer average total numbers (4 ± 2 cells/mg tissue) and percentage (0.25%) of basophils than either NP subgroup (Figure 1D and data not shown). However, we found no significant difference in number of nasal polyp basophils based on the presence or absence of atopy (data not shown). Among all patients with NP (both CRSwNP and AERD), the number of basophils significantly correlated with the number of eosinophils ($r = 0.64$; $p < 0.001$) (Figure 1E) and the number of mast cells ($r = 0.56$; $p < 0.001$) (Figure 1F). These findings suggest that NP in both AERD and CRSwNP are characterized by a type 2 inflammatory environment and that the mechanisms of expansion of these three cell types may be overlapping.

Identification of type 2 inflammatory granulocytes in peripheral blood

We next examined the peripheral blood of control subjects and patients with AERD or CRSwNP to determine if there were similar elevations in circulating inflammatory cells. Interestingly, AERD patients on average had significantly more peripheral leukocytes ($4,257 \pm 364$ cells/ μ L

blood) than CRSwNP patients ($2,777 \pm 455$ cells/ μ L blood; $p < 0.01$, Mann-Whitney) and controls ($2,967 \pm 410$ cells/ μ L blood; $p = 0.01$ Kruskal-Wallis) (Figure 2A). In contrast, the number of circulating leukocytes of CRSwNP patients was not different from that of controls (Figure 2A). The average number of peripheral eosinophils was also higher in AERD (242 ± 57 cells/ μ L blood) than CRSwNP (155 ± 37 cells/ μ L blood) but this did not reach statistical significance ($p = 0.36$) (Figure 2B).

Basophils were significantly elevated in the circulation of AERD (38 ± 8 cells/ μ L blood) compared to CRSwNP patients (17 ± 3 cells/ μ L blood; $p = 0.04$) (Figure 2C). When data for the number of basophils and the number of eosinophils in the peripheral blood of AERD and CRSwNP patients were combined and compared, a strong correlation was observed between these two cell types ($r = 0.64$; $p = 0.0001$) (Figure 2D).

As tissue basophils are recruited from the periphery, we next assessed if there was a significant correlation between the number of basophils in the peripheral blood and the number of basophils in NP in matched individuals with CRSwNP or AERD. When assessing both conditions together, we found a significant correlation between basophils in NP and blood ($r = 0.44$; $p = 0.02$) (Figure 3A). However, this correlation was driven primarily by patients with AERD as we found no significant correlation between tissue basophils and peripheral basophils in patients with just CRSwNP ($r = 0.32$; $p = 0.29$) (Figure 3B). In contrast, a stronger correlation was observed when assessing only AERD patients ($r = 0.52$; $p = 0.07$) (Figure 3C).

Assessment of basophil activation

We next assessed the activation status of basophils in CRSwNP and AERD by evaluating the surface expression of traditional basophil activation markers, CD203c and CD63, using flow cytometry. We found no difference in the geometric mean fluorescence intensity (MFI) of CD203c on circulating basophils of patients with AERD (939 ± 141), CRSwNP (821 ± 103), or controls ($1,279\pm216$) (Figure 4A). In contrast, CD203c MFI was significantly upregulated on basophils derived from NP compared to those from peripheral blood in both AERD ($2,290\pm294$ vs 939 ± 141 ; $p=0.001$) and CRSwNP ($1,805\pm123$ vs 821 ± 103 ; $p=0.001$) (Figure 4A). There was no significant difference in CD203c MFI on basophils from NP of AERD versus CRSwNP ($p>0.99$).

We observed similar findings when measuring another basophil activation marker, CD63. The average levels of CD63 were comparable on circulating basophils in control (989 ± 200), CRSwNP ($1,202\pm221$), and AERD ($1,121\pm189$) patients (Figure 4B). However, CD63 was significantly upregulated on basophils in NP compared to the circulation in both AERD ($p<0.001$) and CRSwNP ($p=0.003$). Levels of CD63 on NP basophils were similar between CRSwNP and AERD ($p>0.99$) (Figure 4B). We also quantified the absolute number of basophils that highly expressed CD63. In NP, there were significantly more CD63^{hi} basophils in AERD (25 ± 8 cells/mg tissue) than CRSwNP (6 ± 1 cells/mg tissue, $p=0.002$) (Figure 4C). Basophil activation appears to be restricted to the tissue, as there were minimal CD63^{hi} basophils detected in the blood, and no differences seen between control, CRSwNP, and AERD (Figure 4D). Taken together these findings suggest that basophils become activated similarly in CRSwNP and AERD only once they enter NP. Although the level of activation is equivalent (as measured by CD63 and CD203c MFI) in both

disease conditions, the number of highly activated basophils in NP tissue is higher in AERD than CRSwNP.

Assessment of basophil degranulation

The antibody 2D7 is well-established and specifically labels basophil secretory granules²¹. As mentioned above, we previously observed that 2D7 staining in NP via immunohistochemistry was lower in AERD compared to CRSwNP¹⁰. We hypothesized that basophils in AERD had degranulated to a greater extent in AERD, leading to lower levels of 2D7 and reduced detection by immunohistochemistry. To test this hypothesis, we utilized flow cytometry to measure intracellular levels of 2D7 in basophils from patients with AERD and CRSwNP.

The MFI of 2D7 was the highest in circulating basophils and there were no significant differences in 2D7 observed between controls ($5,690 \pm 1,263$) and patients with CRSwNP ($9,668 \pm 1,652$; $p=0.74$) or AERD ($7,728 \pm 1,570$) (Figure 5A). In contrast, 2D7 MFI levels in NP basophils were reduced in AERD ($1,522 \pm 384$) compared to CRSwNP ($4,742 \pm 1,073$; $p=0.06$) (Figure 5A). Levels of 2D7 were lower in basophils isolated from NP compared to the peripheral blood in AERD patients ($p<0.001$) but not in CRSwNP patients ($p=0.28$), suggesting that basophil degranulation may be occurring to a greater extent in NP of AERD versus CRSwNP (Figure 5A).

In addition to measuring the intensity of 2D7 staining (MFI), we assessed how many basophils expressed 2D7 out of the total basophil population (the percent positive). In the circulation of all subject groups, nearly all basophils expressed 2D7 (Figure 5B). In contrast, significantly fewer

NP basophils expressed 2D7 in AERD (33%) compared to CRSwNP (65%; $p=0.005$) (Figure 5C). These findings suggest that basophils have degranulated and released 2D7 to a greater extent in AERD NP and provide a mechanistic explanation for our previous observation of reduced levels of 2D7 positive cells in AERD polyps¹⁰.

Association with Clinical Disease Severity

To assess the relevance of basophil recruitment, activation, and degranulation to sinus disease across the spectrum of nasal polyposis disease severity, we evaluated whether markers of basophil activation and degranulation were associated with clinical disease severity. The Lund-Mackay (LM) score is a radiographic assessment of sinonasal inflammation visible on sinus CT scan, with a higher score representing more severe disease. We found a significant correlation between the number of CD63^{hi} basophils detected in NP and LM score ($r=0.39$; $p=0.02$) (Figure 6A). Additionally, the number of CD63^{hi} basophils inversely correlated with FEV1 ($r=-0.42$, $p=0.04$), that is to say that patients with more activated basophils had lower lung function and more severe lower airway disease (Figure 6B). Furthermore, there was a significant inverse correlation between basophil 2D7 MFI and LM score ($r=-0.56$; $p=0.004$) (Figure 6C) and a significant positive correlation between 2D7 MFI and FEV1 ($r=0.62$; $p=0.02$) (Figure 6D). This suggests basophil degranulation (and activation) is associated with more severe upper and lower respiratory clinical disease as measured by radiography and pulmonary function testing respectively.

Discussion

To our knowledge, this is the first study to functionally characterize basophils in NP of AERD and CRSwNP patients using flow cytometry or other methods. Basophils were more numerous in NP compared to healthy sinonasal tissue, and the total number of basophils and the proportion of activated (CD63^{hi}) basophils were highest in AERD compared to CRSwNP. This is also the first to quantitatively evaluate expression of the basophil granule marker 2D7 in diseased tissue. We found a lower percentage of 2D7⁺ basophils and reduced 2D7 intensity on basophils in NP of AERD compared to CRSwNP. Based on 2D7 levels in peripheral blood basophils and total basophil counts in tissues, we hypothesize that the reduction in 2D7 likely represents degranulation of basophils within NP tissue. The degree of basophil degranulation, as determined by loss of 2D7, significantly correlated with enhanced sinus and asthma disease severity, measured by Lund-MacKay score and FEV1 respectively. Our findings support the hypothesis that basophils are recruited to NP in AERD and are subsequently activated to release granular contents, suggesting that they may contribute to clinical disease.

In addition to increased basophil numbers in NP, we also found elevated basophils in the blood of AERD patients. This novel observation may be secondary to our assessment of peripheral basophils using flow cytometry. In contrast, prior studies examining peripheral basophils utilized automated cell counters available in clinical laboratories^{22,23}. Unfortunately, we are unable to directly compare the number of basophils assessed by flow cytometry with automated counts since routine blood counts with differentials were not drawn on the day of sinus surgery. Nonetheless, absolute basophil counts were available from some of our study

patients prior to their sinus surgery. Comparison of this limited number of tests suggested no significant difference in circulating basophil numbers between AERD or CRSwNP patients by automated count ($p>0.99$). We suspect the higher sensitivity of the flow cytometry-based approach explains the difference in results.

The underlying pathophysiological mechanisms leading to the elevated number of basophils in both the circulation as well as NP in AERD is unknown. Greater production, longer survival, and/or reduced exit from the circulation could all theoretically result in increased total numbers of basophils in the circulation of AERD patients. Reduced exit seems less likely based on the increased numbers of basophils we observed in NP. However, more basophil progenitors could be produced in the bone marrow and/or basophil progenitors in the bone marrow could have a longer productive life and produce greater numbers of basophils in AERD. The sinonasal tissue could also generate basophil stimulating factors that work locally or activate the marrow remotely to generate more cells^{24,25}. IL-3 is an important cytokine for basophil production and studies evaluating levels in the circulation or bone marrow in AERD would be of interest.

Differential expression of various chemotactic factors may also contribute to the elevated numbers of NP basophils observed in AERD. Eotaxin-1, eotaxin-2, and eotaxin-3 are well-established basophil chemoattractant factors²⁶. Compared to healthy sinonasal tissue, these chemokines are significantly elevated in NP, although no difference was observed between AERD and CRSwNP²⁷. While eotaxins likely contribute to some degree of baseline recruitment, we hypothesize that other mediators may be involved in the selective recruitment of basophils

in AERD. Since basophils express the PGD₂ receptor CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), it is possible that basophils are recruited by elevated levels of PGD₂ known to selectively occur in AERD^{12,13}. It is also possible that the quantity of basophils recruited in AERD is similar to that recruited in CRSwNP but that basophils survive longer in AERD NP as a result of local survival factors.

In addition to measuring the total number of basophils, we also evaluated their activation status. CD63 is a marker of degranulation as it is exposed on the cell surface upon the fusion of the basophil granular and surface membranes. In our study, CD63 MFI was significantly upregulated on NP basophils in both CRSwNP and AERD compared to peripheral blood. In contrast, CD203c is a surface marker associated with basophil activation but not necessarily degranulation^{28,29}. We found CD203c MFI was elevated on NP basophils in both CRSwNP and AERD compared to peripheral blood. MacGlashan has previously reported that upregulation of CD203c and CD63 expression on basophils may vary based on the type of stimulus and mode of degranulation (piecemeal versus anaphylactic)³⁰. However, the mechanism by which basophils are activated in NP remains unclear.

IgE remains one of the most well-known activators of basophils. However, in contrast to atopic conditions such as allergic rhinitis or asthma, neither CRSwNP nor AERD is thought to be due to conventional antigen driven IgE-mediated hypersensitivity reactions. However, previous work from our group and others found elevated local production of IgE in NP compared to the

circulation of matched subjects or to healthy sinonasal tissue^{31,32}. It is thus possible that local IgE is responsible in part for activating basophils in NP.

Alternatively, basophils could be activated through non-IgE mediated pathways. Various chemokines (e.g. monocyte chemotactic proteins 1-3, eotaxins 1-3, RANTES), proteases (which activate basophils through PAR receptors), formyl methionine peptides (which activate basophils through specific G protein coupled receptors), and complement split products (C3a, C5a) should be evaluated as other potential activating factors. C3a and C5a are especially intriguing given that we and others have reported elevated levels of these products in CRS^{33,34}. It may also be that basophils are more selectively primed by cytokines like IL-3 in AERD and thus are more easily activated by the same level of stimuli. Future studies investigating the mechanisms of production, survival, recruitment, priming, and activation of basophils in AERD (and CRSwNP) are needed.

We also assessed the intracellular expression with 2D7, an antibody that labels a secretory granule protein found in basophils²¹. Previously, we reported a reduction in the number of 2D7⁺ basophils in NP of AERD compared to CRSwNP by immunohistochemistry¹⁰. This observation was confirmed by our current studies showing that basophils expressed less 2D7 (both as a percentage and an average relative intensity) in NP from AERD patients compared to either matched blood or NP from patients with CRSwNP. We hypothesize that basophils in AERD NP lose their 2D7 staining upon degranulation, thus making basophils more difficult to enumerate. We had initially expected that 2D7 MFI would inversely mirror that of CD63 MFI (since both are

associated with degranulation). However, unlike 2D7 MFI, we did not see any difference in CD63 MFI between NP basophils from CRSwNP and AERD patients, nor was there any significant correlation between CD63 and 2D7 MFI (data not shown). This dissociation of the two biomarkers may be due to differences in dose response or time course for 2D7 loss versus CD63 upregulation; alternatively, it is possible that CD63, once transported to the cell surface, may subsequently undergo further processing (e.g. shedding, internalization, cleavage, etc). Further work is thus needed to understand the molecular basis for the dissociation of 2D7 down-regulation and CD63 upregulation associated with basophil degranulation.

While the biological significance of 2D7 remains to be fully elucidated, we found a significant correlation between loss of 2D7 intensity and upper and lower respiratory disease severity as measured by greater opacification on sinus CT and reduced pulmonary function. This is the first reported association, to our knowledge, between 2D7 and clinical disease severity. However, our findings support the conclusions of Brescia and colleagues, who found that CRSwNP patients who had peripheral basophil counts greater than 0.03×10^9 cells/L had higher rates of sinonasal disease recurrence²³.

It is possible that the granule protein identified by 2D7 contributes to AERD pathogenesis in yet-to-be-discovered mechanisms. Activated basophils produce IL-4 and IL-13, which can stimulate type 2 inflammatory responses including mucus hypersecretion, induction of IgE class switching, and impairment in the epithelial barrier, all known to be dysregulated in NP³⁵. Additionally, basophils are potent producers of cysteinyl leukotrienes which are known to be

elevated in AERD^{14,15}. Interestingly, we recently identified a novel lipid mediator, 15-oxo-EETE, in AERD NP that is generated through the conversion of arachidonic acid via the 15-lipoxygenase and hydroxyprostaglandin dehydrogenase (HPGD) enzymes³⁶. Mast cells³⁶ and basophils³⁷ (<http://www.proteinatlas.org>) both highly express HPGD and it is thus possible that basophils are an important source of 15-oxo-EETE.

A limitation of this study is that 45% and 64% of CRSwNP and AERD patients respectively were taking oral corticosteroids peri-operatively and this may have affected basophil numbers. To address this, we examined basophils numbers in both conditions according to steroid use. We found no difference in basophil numbers in NP of either AERD or CRSwNP when stratified by history of intranasal, inhaled, or systemic corticosteroid use (Supplemental Figure 4). Furthermore, Lampl and colleagues reported in the last millennium that chronic treatment with steroids in patients with severe asthma or connective tissue disorders led to a resistance in the *in vitro* inhibitory effects of dexamethasone on basophils³⁸.

In this study, AERD patients had not undergone an aspirin desensitization nor were they taking a daily high-dose aspirin regimen. In work by Cahill et al, peripheral basophil numbers increased in AERD patients who had been successfully desensitized and maintained on high-dose aspirin for 8 weeks³⁹. This increase in peripheral basophils may represent a reduction in basophil recruitment to NP and thus potentially reflect the underlying mechanism by which patients experience clinical improvement.

432 In summary, basophils play an important role in promoting a type 2 inflammatory response in a
433 variety of allergic conditions. This is the first study to report significantly elevated numbers and
434 activation of basophils in NP compared to healthy sinonasal tissue. AERD patients had
435 particularly high elevations of basophils not only in their NP but also in their peripheral blood.
436 While basophils are activated in NP of both CRSwNP and AERD, basophils appear to have
437 degranulated to a greater extent in AERD. Levels of basophil recruitment and activation
438 showed clear correlations with the physiological and pathological measures of disease severity,
439 FEV1 and Lund-MacKay score.

440 **Acknowledgements**

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442 antibody used to identify basophils by immunohistochemistry in Supplemental Figure 3.

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Figure Legends**Figure 1. Basophils are elevated in nasal polyps of patients with AERD.**

Flow cytometry was used to quantify the number of CD45⁺ leukocytes (A), CD45⁺ Siglec-8⁺ FcεRI⁻ eosinophils (B), CD45⁺ CD117⁺ FcεRI⁺ mast cells (C), and CD45⁺ CD203c⁺ FcεRI⁺ CD117⁻ basophils (D) in nasal polyps of patients with CRSwNP or AERD. For comparison, the number of leukocytes (A) and basophils (D) were quantified in inferior turbinate tissue of healthy controls. The number of tissue basophils strongly correlated with the number of tissue eosinophils (E) and tissue mast cells (F) in nasal polyps. Dot plots illustrate individual data points with solid lines representing the median and interquartile range. Statistical significance ($p < 0.05$) was determined by Mann-Whitney U test comparing AERD and CRSwNP (A-D) or Spearman's rank correlation (E-F); * $p < 0.05$.

Figure 2. Basophils are elevated in the peripheral blood of patients with AERD.

Flow cytometry was used to quantify the number of CD45⁺ leukocytes (A), CD45⁺ Siglec-8⁺ FcεRI⁻ eosinophils (B), and CD45⁺ CD203c⁺ FcεRI⁺ CD117⁻ basophils (C) in the peripheral blood of patients with CRSwNP or AERD or healthy controls. The number of peripheral blood basophils strongly correlated with the number of circulating eosinophils (D) in CRSwNP and AERD patients combined. Dot plots illustrate individual data points with solid lines representing the median and interquartile range. Statistical significance ($p < 0.05$) was determined by Mann-Whitney U test comparing AERD and CRSwNP (A-C) or Spearman's rank correlation (D); * $p < 0.05$, ** $p < 0.01$.

Figure 3. Basophil numbers strongly correlated between the peripheral blood and nasal polyps of patients with AERD but not in those with CRSwNP.

The number of CD45⁺ CD203c⁺ FcεRI⁺ CD117⁻ basophils in matched peripheral blood and nasal polyps were compared when patients with AERD or CRSwNP were combined (A) or when CRSwNP (B) and AERD (C) patients were analyzed separately. Dot plots illustrate individual data points, with blue representing a CRSwNP patient and black representing a patient with AERD. Statistical significance ($p < 0.05$) was determined by Spearman's rank correlation.

Figure 4. Basophils expressed higher levels of activation markers CD63 and CD203c in nasal polyps compared to peripheral blood in both AERD and CRSwNP.

Flow cytometry was used to quantify the mean fluorescence intensity (MFI) of CD203c (A) and CD63 (B) on basophils isolated from the peripheral blood and from nasal polyps of patients with AERD or CRSwNP. Peripheral blood from healthy controls was used for comparison. The number of CD63^{hi} basophils was quantified in nasal polyps (C) and peripheral blood (D) from the diseased and healthy conditions. Dot plots illustrate individual data points with solid lines representing the median and interquartile range. Statistical significance ($p < 0.05$) was determined by Kruskal-Wallis test with post-hoc analysis using the Dunn's test for multiple comparison (A-B) and by Mann-Whitney U test comparing AERD and CRSwNP (C-D); ** $p < 0.01$, *** $p < 0.001$.

Figure 5. Basophils have reduced levels of 2D7 expression in nasal polyps of patients with AERD compared to those with CRSwNP.

Flow cytometry was used to quantify the mean fluorescence intensity (MFI) of intracellular 2D7 expression (A) within basophils isolated from the peripheral blood and from nasal polyps of patients with AERD or CRSwNP. Peripheral blood from healthy controls was used for comparison. The percentage of basophils expressing 2D7⁺ in peripheral blood (B) and nasal polyps (C) was also determined. Dot plots illustrate individual data points, with solid lines representing the median and interquartile range. Statistical significance ($p<0.05$) was determined by Kruskal-Wallis test with post-hoc analysis using the Dunn's test for multiple comparison (A) and by Mann-Whitney U test comparing AERD and CRSwNP (B-C); * $p<0.05$, *** $p<0.001$.

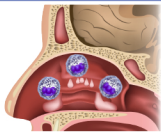
Figure 6. Markers of basophil activation (CD63) and degranulation (2D7 MFI) correlated with sinonasal and pulmonary disease severity.

The number of CD63^{hi} basophils significantly correlated with Lund-Mackay score (A) and inversely correlated with Fraction of Exhaled Volume in 1 second (FEV1) (B) in patients with either CRSwNP or AERD. Additionally, 2D7 MFI inversely correlated with Lund-Mackay score (C) and positively correlated with FEV1 (D) in both CRSwNP and AERD. Dot plots illustrate individual data points, with blue representing a CRSwNP patient and black representing a patient with AERD. Statistical significance ($p<0.05$) was determined by Spearman's rank correlation.

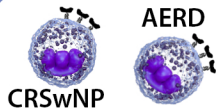
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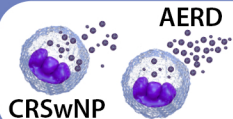
Basophils are elevated in nasal polyps of patients with Aspirin Exacerbated Respiratory Disease and may contribute to disease pathogenesis



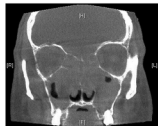
Basophils are elevated in NP and peripheral blood in AERD compared to CRSwNP



NP basophils have similar levels of CD63 activation in AERD and CRSwNP



Basophils in AERD NP have degranulated more than in CRSwNP as judged by loss of 2D7⁺ granules



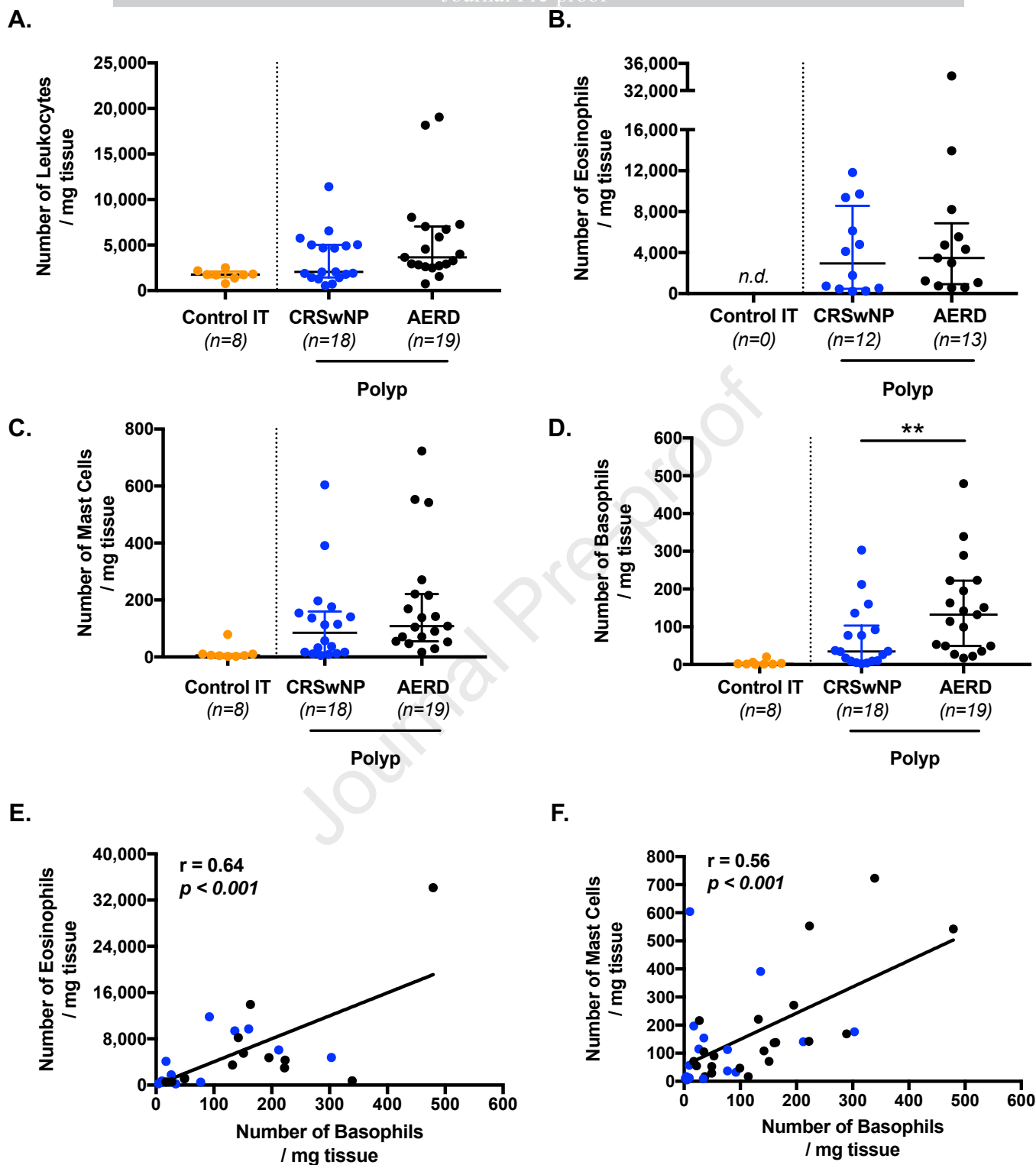
Extent of basophil degranulation correlates with enhanced sinonasal disease severity

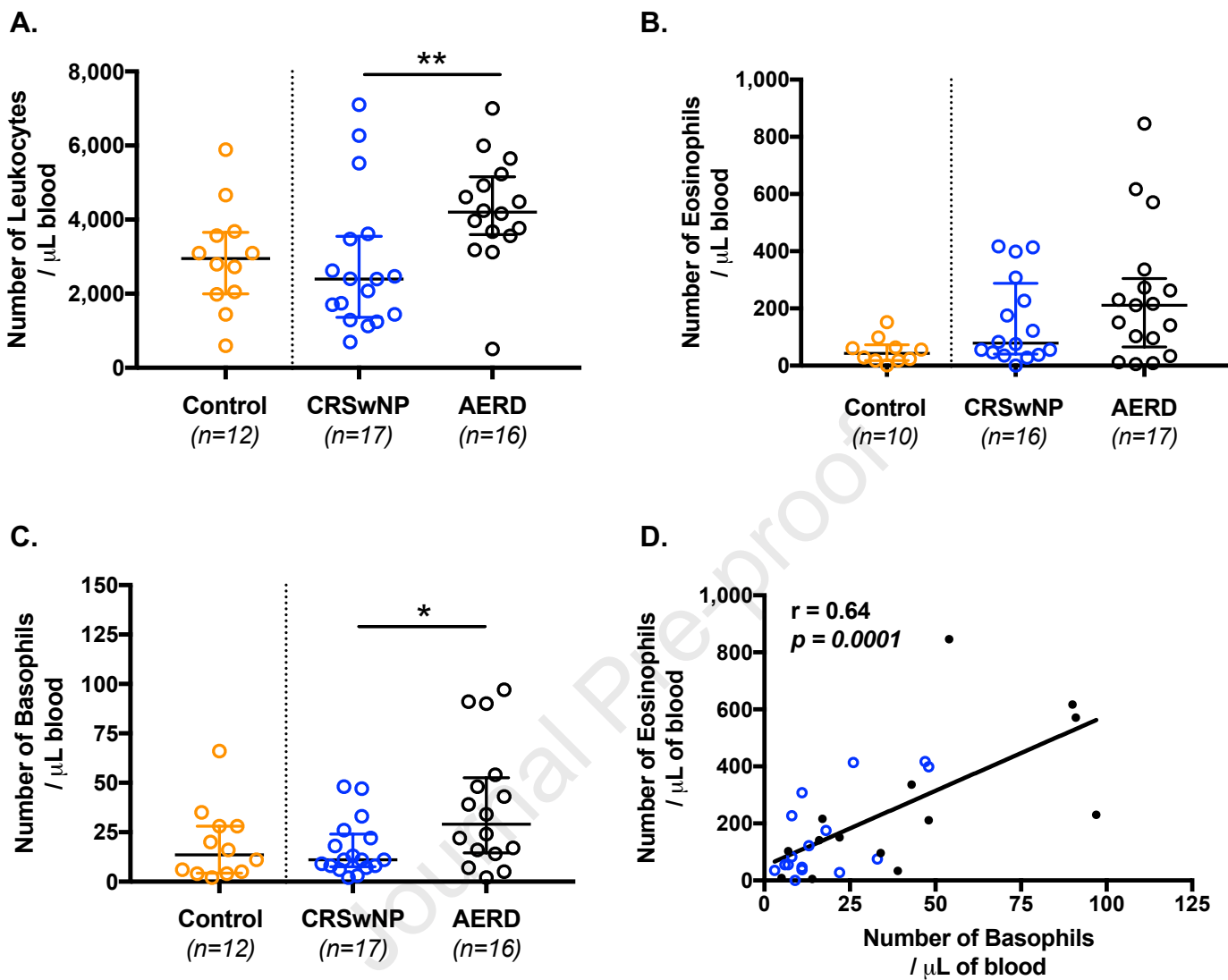
Abbreviations:

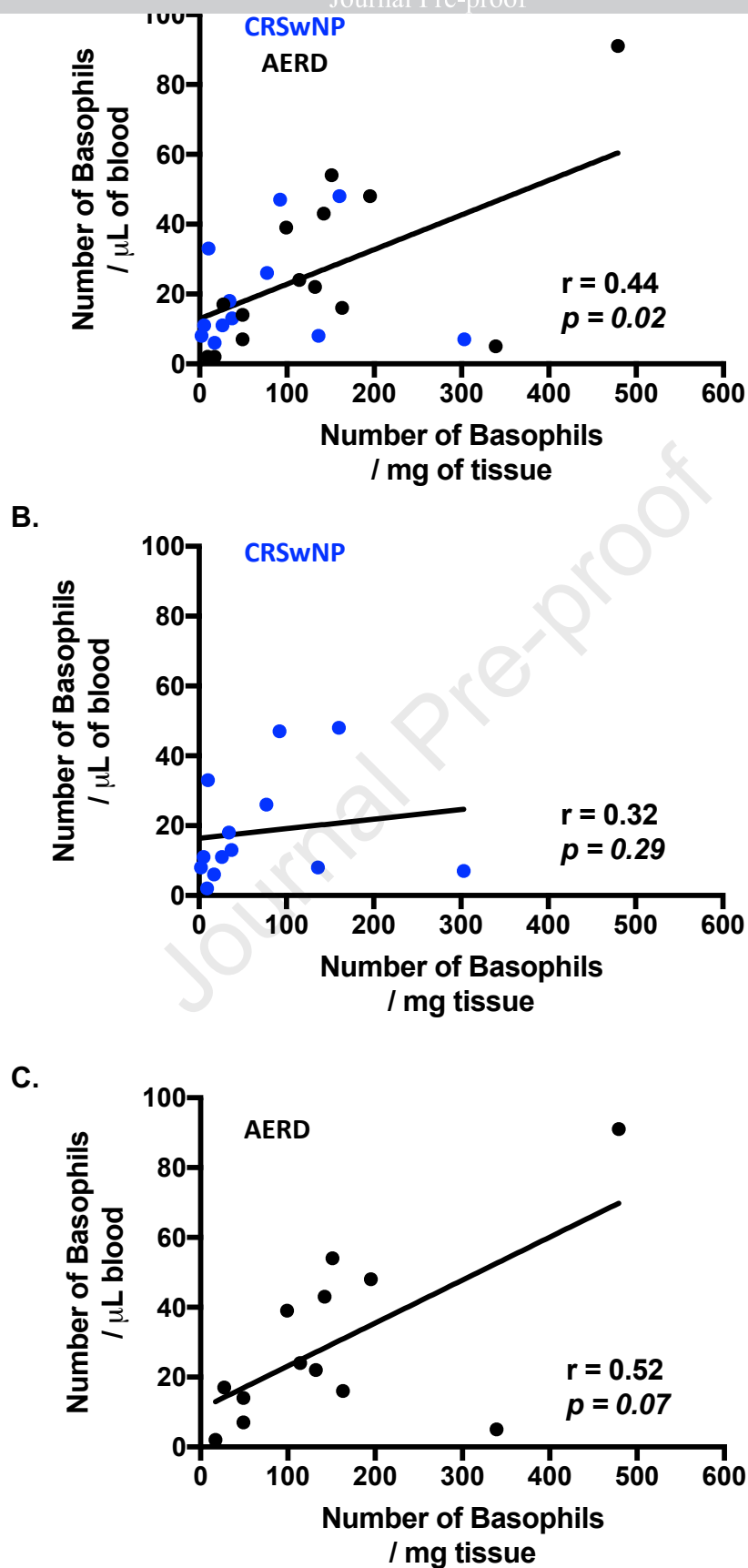
AERD - Aspirin Exacerbated Respiratory Disease

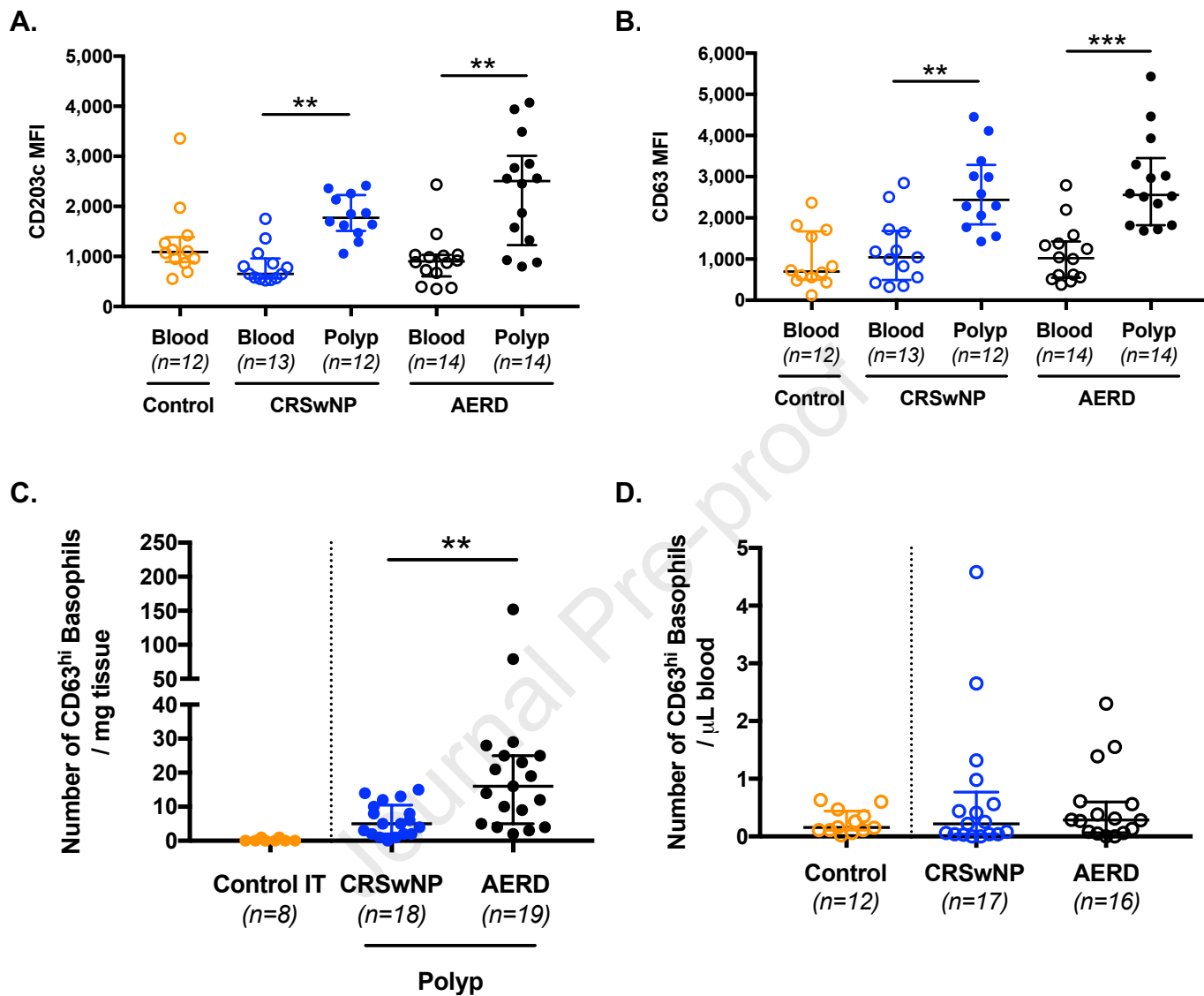
CRSwNP - Chronic rhinosinusitis nasal polyps

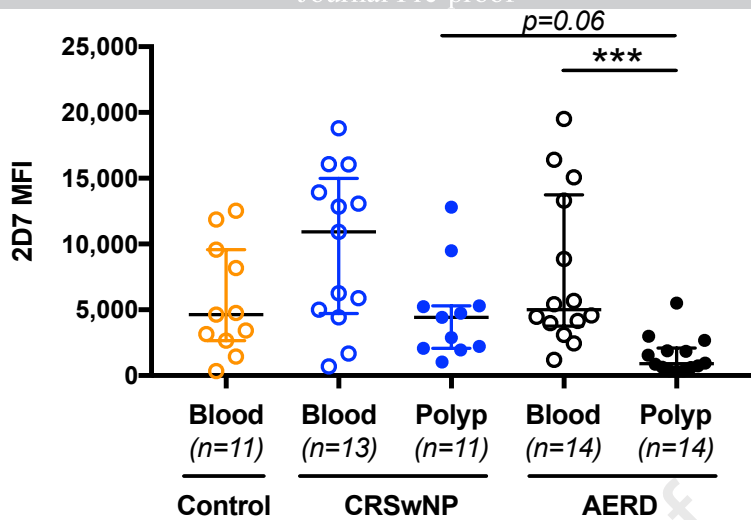
NP - Nasal polyp



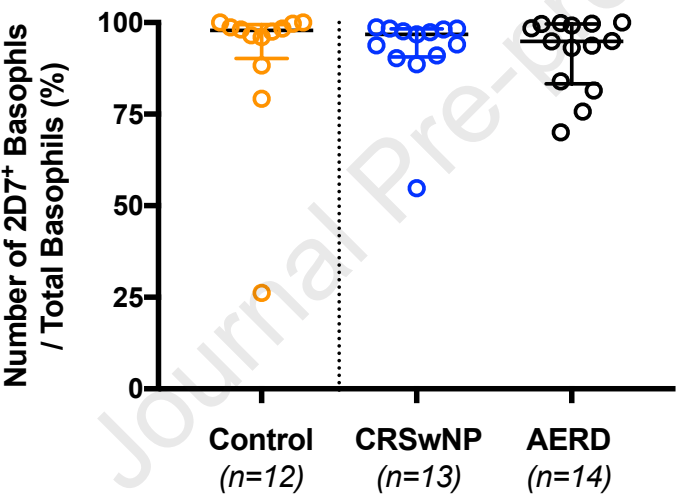








B.



C.

