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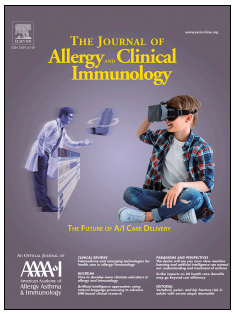

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Running Title: Inflammatory heterogeneity in AERD

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ABSTRACT

Background: Aspirin-exacerbated respiratory disease (AERD) is a mechanistically distinct subtype of chronic rhinosinusitis with nasal polyps (CRSwNP). Though frequently associated with type 2 inflammation, literature characterizing the milieu of inflammatory cytokines and lipid mediators in AERD has been conflicting.

Objective: To identify differences in the upper airway inflammatory signature between CRSwNP and AERD and determine whether endotypic subtypes of AERD may exist.

Methods: Levels of 7 cytokines representative of type 1, type 2, and type 3 inflammation, and 21 lipid mediators were measured in nasal mucus from 109 patients with CRSwNP, 30 patients with AERD, and 64 non-CRS controls. Differences in inflammatory mediators were identified between groups and patterns of inflammation among AERD patients were determined by hierarchical cluster analysis.

Results: AERD could be distinguished from CRSwNP by profound elevations in IL-5, IL-6, IL-13, and IFN-γ, however, significant heterogeneity existed between patients. Hierarchical cluster analysis identified three inflammatory sub-endotypes of AERD characterized by 1) low inflammatory burden, 2) high type 2 cytokines, and 3) comparatively low type 2 cytokines and high levels of type 1 and type 3 cytokines. Several lipid mediators were associated with asthma and sinonasal disease severity, however, lipid mediators showed less variability than cytokines.

Conclusion: AERD is associated with elevations in type 2 cytokines (IL-5, and IL-13) and the type 1 cytokine, IFN-γ. Among patients with AERD, the inflammatory signature is heterogeneous, supporting sub-endotypes of the disease. Variability in AERD immune signatures should be further clarified as this may predict clinical response to biologic medications that target type 2 inflammation.

CAPSULE SUMMARY:

The upper airway immune signature in AERD is heterogeneous with variable levels of type 1, type 2, and type 3 immune mediators, suggesting sub-endotypes within AERD.

CLINICAL IMPLICATIONS:

AERD is homogenous with respect to lipid mediators but has variable levels of type 1, type 2, and type 3 cytokines, suggesting likely variability in response to therapeutics that target type 2 inflammation.

ABBREVIATIONS

AA: arachidonic acid
AERD: aspirin-exacerbated respiratory disesase
BMI: body mass index
COX-1: cyclo-oxygenase-1
CRSwNP: chronic rhinosinusitis with nasal polyps
CRTH2: choemoattractant receptor-homologous molecule expressed on Th2 cells
CT: computed tomography
CV: coefficient of variation
CysLT: cysteinyl leukotrienes
GINA:
IFN: interferon
IL: interleukin
IQR: interquartile range
LA: linoleic acid
LT: leukotriene
LTR: leukotriene modifier
NCS: nasal corticosteroid
NSAID: non-steroidal anti-inflammatory drug
PG: prostaglandin
SNOT-22: 22 item sinonasal outcome test
UPLC: ultra-performance liquid chromatography
TNF: tumor necrosis factor
TP: thromboxane receptor
Tx: thromboxane
5-LO: 5-lipoxygenase

Key Words: cytokine, endotype, leukotriene, prostaglandin, aspirin, NSAID, rhinosinusitis, eicosanoid, asthma

INTRODUCTION

Chronic Rhinosinusitis (CRS) is a common but heterogeneous airway inflammatory disease that affects up to 5% of the U.S. population[1, 2]. Though mechanisms of disease are still being determined, it is now increasingly recognized that CRS likely represents a clinical syndrome with potentially diverse pathophysiology rather than a single diagnosis. Unique phenotypes and endotypes of CRS are now being characterized based on clinical presentation and inflammatory characteristics, respectively, and these subtypes have important implications for disease management and prediction of clinical outcomes. Aspirin-exacerbated respiratory disease (AERD) is a well-established disease subtype characterized by asthma, nasal polyposis, and sensitivity to cyclooxygenase-1 (COX-1) inhibitors. When compared with CRSwNP more broadly, AERD is associated with need for more surgery and patients are more likely to be corticosteroid dependent[3, 4].

Though knowledge of the exact pathogenesis of nasal polyposis is unclear and ever evolving, the inflammatory environment associated with polyps in Western countries is usually eosinophilic, with a predominantly type 2 immune signature[5-7]. The AERD subtype of CRSwNP has a similar type 2 predominance but has unique biochemical hallmarks that distinguish it from other patients with nasal polyposis. For example, AERD is commonly associated with degranulated mast cells and excess production of cysteinyl leukotrienes (cysLTs) generated from arachidonic acid (AA) by the 5-lipoxygenase (5-LO) pathway. Nonetheless, recent investigations suggest that AERD may not be a singular disease process but rather a heterogeneous entity with substantial differences in inflammatory mediators and clinical characteristics. A recent Belgian analysis of a large AERD cohort identified three potential “subphenotypes” of AERD with variability among measured inflammatory mediators in blood and induced sputum, and with respect to multiple demographic and clinical characteristics[8]. While the characteristic cytokine milieu of AERD is notable for a predominantly type 2-dominant inflammatory profile, precise characterization of this signature has varied considerably in the literature, particularly with respect to the upper airway. Perez-Novo previously found that IL-5 was elevated in sinonasal tissue from AERD patients compared to patients with CRSwNP[9]. However, Steinke et al. reported that polyps from

patients with AERD had reduced levels of the prototypical type 2 cytokines IL-5 and IL-13 compared to those with hyperplastic eosinophilic CRSwNP, while IL-4 and the type 1 cytokine IFN-γ were increased[10]. Conversely, Stevens et al. found no differences in IFN-γ, IL-5, or IL-13 between CRSwNP and AERD polyps, and subsequently proposed that eosinophilic inflammation in AERD may be mediated by factors other than traditional type 2 cytokines[11]. Recent identification of putative CRS endotypes, including work by our group, have confirmed that CRSwNP is a complex disease characterized by contributions from multiple different inflammatory mediators, rather than by a purely type 2 cytokine milieu[12-14]. Interestingly, hierarchical cluster analysis of CRS patients by our group also found that patients with AERD do not converge within a single inflammatory endotype, and were instead irregularly distributed among 4 out of 5 endotypic clusters using this methodology[13]. Histologic classification based on granulocytic tissue infiltration similarly showed that AERD can present with mixed eosinophilic and neutrophilic inflammation in up to 30% of patients, with some characterized by neutrophilic inflammation alone, despite being universally linked to type 2 inflammation[15].

Given these discordant findings and suggestions of multiple inflammatory pathways, we hypothesized that there may be endotypic features within the greater AERD subtype that could have significant clinical relevance. We sought to identify any differences in inflammatory mediators in the upper airway between CRSwNP and AERD, and further sought to identify any inflammatory AERD subgroups using an unstructured approach. Finally, we determined whether these inflammatory AERD subgroups were linked to differences in CysLTs and other lipid mediators associated with AA and linoleic acid (LA) metabolism.

METHODS

Study Population

This study was approved by the Institutional Review Board of Vanderbilt University. Patients presented to Rhinology clinics at the Vanderbilt Bill Wilkerson Center and Vanderbilt Asthma, Sinus, and

Allergy Program. CRS was diagnosed according to the European Position Paper on Rhinosinusitis and Nasal polyps and International Consensus Statement on Allergy and Rhinology[16, 17]. CRSwNP was diagnosed by the presence of visible nasal polyps on clinic nasal endoscopy or during endoscopic sinus surgery. AERD was diagnosed based on the clinical triad of nasal polyps, asthma, and at least two documented allergic reactions to aspirin or other NSAIDs or a positive aspirin challenge. All patients underwent endoscopic sinus surgery after failing a period of medical management. Control cases were patients undergoing anterior skull base or pituitary surgery without clinical or radiographic history of sinus disease. Exclusion criteria included any patient receiving systemic steroids within 4 weeks prior to surgery, patients with cystic fibrosis, autoimmune, or granulomatous disease or patients who were receiving immune directed monoclonal antibodies or other immunomodulators. The presence of asthma and allergic rhinitis was recorded for all patients. Allergic rhinitis was diagnosed based on positive skin prick testing or physician diagnosis based on seasonal variation in atopic symptoms and relief after using an oral antihistamine or intranasal corticosteroids. Asthma was diagnosed based on a bronchodilator response on pulmonary function testing, methacholine challenge, or prior diagnosis by a pulmonologist and/or allergist. Asthma severity was defined by asthma medication use at the time of surgery based on GINA guidelines. The Sinonasal Outcome Test-22 (SNOT-22) was used to record patient reported symptom severity[18]. All patients underwent high-resolution CT scan of the paranasal sinuses within 3 months of surgery. A Lund McKay score was assigned to each scan to assess the severity of radiographic sinus disease.

Mucus Collection and Cytokine Measurement

9 × 24mm polyurethane sponges (Summit Medical; St. Paul, MN) were placed into the middle meatus or ethmoid cavity of each subject under endoscopic guidance at the time of surgery, as has been previously reported[19, 20]. This method of mucus collection has the advantage of minimal specimen dilution and standardization between patients, and we have previously shown that mucus cytokine levels are highly consistent within different subsites of the sinonasal cavity[21]. Sponges were left in place for 5

minutes, after which they were placed in a sterile microcentrifuge tube and immediately processed. Sponges were placed into a microporous centrifugal filter device (MilliporeSigma; Billerica, MA) and centrifuged at 14,000 × g for 10 minutes. Samples were then gently vortexed and again centrifuged for 5 minutes to remove cellular debris. Supernatants were removed, placed into a new microcentrifuge tube, and frozen at −80°C.

A multiplex cytokine bead assay (BD Biosciences; Franklin Lakes, NJ) was then used to analyze samples. In brief, 50 μL of mucus was incubated with 50 μl of mixed capture beads for each measured inflammatory mediator and incubated for 1 hour. 50 μL of mixed detection reagent was then added to each sample and standard and incubated for an additional 2 hours. Samples were centrifuged at 200 × g for 5 minutes after the addition of 1 mL wash buffer, and the supernatant was discarded. The beads were then resuspended in 300 μL wash buffer and analyzed on an LSR Fortessa flow cytometer (BD Biosciences; San Jose, CA). A total of seven cytokines and inflammatory mediators were analyzed. The assay data was analyzed using BD FCAP Array Software version 3.0.

Measurement of Eicosanoids

AA and LA-derived lipid mediators detectable by our ultra-performance liquid chromatography (UPLC)-mass spectroscopy approach were assessed in the mucus samples collected at the time of surgery (Figure S1). 25-40uL of each mucus specimen was placed into a microcentrifuge tube containing 5000uL 25% methanol in water and internal standard mix (1ng each deuterated eicosanoid). The sample was vortexed and spun to pellet protein. The supernatant was then extracted on an Oasis MAX uElution plate (Waters Corp., Milford, MA) as follows: Sample wells were first washed with methanol (200uL) followed by 25% methanol in water (200uL). The sample was then loaded into the well and washed with 600uL 25% methanol. Eicosanoids were eluted from the plate with 30uL 2-propanol/acetonitrile (50/50, v/v) containing 5% formic acid into a 96-well elution plate containing 30uL water in each well. Samples were analyzed on a Waters Xevo TQ-XS triple quadrupole mass spectrometer connected to a Waters Acquity I-Class UPLC (Waters Corp., Milford, MA USA). Separation of analytes was obtained using an

Acquity PFP column (2.1 x 100mm) with mobile phase A being 0.01% formic acid in water and mobile phase B acetonitrile. Eicosanoids were separated using a gradient elution beginning with 30% B going to 95% B over 8 minutes at a flow rate of 0.250mL/min.

Statistical Analysis

Descriptive statistics for demographic/clinical characteristics and inflammatory mediators were presented as the median with interquartile (IQR) or mean with standard deviation (SD) for continuous variable and the frequency with percentages for categorical variables. Differences between defined CRS subgroups or clusters were evaluated using the Kruskal-Wallis test for continuous variables or $\chi 2$ test for categorical variables. This was followed by Dunn's test for multiple comparisons. Statistical significance was defined as a P value < 0.05.

Sample size for cluster analysis was estimated by establishing a subject to variable ratio of greater than 5:1 as recommended by Gorsuch and Hatcher[22, 23]. Descriptive statistics and frequency distributions were examined for each biological variable and all were positively skewed. In order to normalize data for subsequent analysis, values were transformed by taking the log-transformation, resulting in elimination or significant reduction of skewing for all variables. Hierarchical cluster analysis was performed using Ward's method on squared Euclidian distances. The hierarchical structure and taxonomic relationships between subjects was visualized using a dendogram. The appropriate number of clusters (k) was selected using the Elbow method. This approach calculates the total within sum of squared error (SSE) for between 1 and 10 clusters and determines k by identifying the break point where adding additional clusters does not substantially change the SSE. Cluster stability was verified using bootstrap analysis. This involved repeating the estimation procedure on 1000 resampled datasets to ensure that the clustering results were stable and not unique to the original dataset. Variances of each biological variable were compared using the coefficient of variation to create a unitless measure of comparison between groups. Omnibus hypothesis testing for differences in variance was performed using the asymptotic test for the equality of coefficients of variation.

RESULTS

Demographics and clinical characteristics

A total of 64 controls and 139 CRS patients were enrolled in the study, including 109 patients with CRSwNP and 30 patients with AERD (Table 1). Patients with AERD and non-AERD CRSwNP were similar with respect to age, gender, and BMI. However, disease burden was generally higher in AERD compared to CRSwNP as assessed by CT score (20.3+/-3.5 vs. 15.9+/- 4.2, $p<0.001$) and SNOT-22 score (50.7±4.2 vs. 42.1+/-2.5, $p=0.08$). Patients with AERD also had higher rhinologic ($p=0.04$) and extranasal ($p=0.01$) SNOT-22 subdomain scores. Patients with AERD were more likely to be on anti-leukotriene medications ($p<0.001$) and were more likely to have had prior endoscopic sinus surgery (70% vs. 36.7%, $p=0.02$).

Cytokine signatures in AERD

Prior studies have reported conflicting results regarding potential AERD-specific inflammatory signatures. To help settle these inconsistencies we assessed five different cytokines measured in sinonasal mucus that are representative of type 1 (IFN-γ), type 2 (IL-4, IL-5, IL-13), and type 3 immunity (IL-17A), as well as two pro-inflammatory cytokines associated with innate immunity (IL-1β, IL-6). As previously reported, CRSwNP and AERD were both characterized predominantly by elevated type 2 cytokines (Table S1). Compared to healthy controls, CRSwNP patients were characterized by elevated IL-4, IL-5, and IL-6, while patients with AERD were characterized by high levels of IL-4, IL-5, IL-6, IL-13, IL-17A, and IFN-γ. AERD could be differentiated from CRSwNP by profoundly higher levels of IL-5, IL-6, IL-13, and IFN-γ, with median values that were 3-5 times greater on average compared to CRSwNP patients (Figure 1). This data confirms that AERD has a predominantly type 2 immune signature, but additionally has features of both type 1 and type 3 inflammation.

Lipid mediator signatures in AERD

We next sought to measure multiple lipid mediators in sinonasal mucus from AERD patients and identify any potential differences compared to CRSwNP and control patients (Table 2). A total of 21 mediators derived from the arachidonic acid and linoleic acid cascades were assessed using (UPLC)-mass spectroscopy. Most metabolites varied significantly between groups, the exceptions being 13-HODE, PGE_2, and LTC_4. AERD could be distinguished from CRSwNP by reduced levels of 9,10-DiHOME (p=0.02), 12,13-DiHOME (p<0.001), 9,10-EpOME (<0.001), 8-HETE (p=0.01), 12-HETE (0.04), 11,12-EET (p<0.001), 14,15-EET (p=0.03), and 20-HETE (p=0.01). TxB_2 was elevated in AERD compared to CRSwNP (p=0.008), while among the cysLTs, LTB_4 levels were reduced (p=0.002) and LTE_4 was increased (p=0.001) (Figure 2).

We then sought to determine whether heterogeneity in inflammatory cytokines was also reflected in levels of lipid mediators with known pathophysiological relevance in AERD. We first compared variability between mucus cytokines and seven lipid mediators in all patients with AERD. We used the coefficient of variation (CV) to make unitless comparisons of variance between the different biomarkers. In general, cytokines had higher variability (IL-1β=1.59; IL-4=1.15; IL-5=1.55; IL-6=1.47; IL-13=1.19; IL-17A=1.25; IFN-γ=4.26) than lipid mediators (LTC_4=0.97; LTE_4=0.86; PGD_2=0.92; PGE_2=0.73; TxB_2=1.13; 20-HETE=0.79), the one exception being LTB_4 (CV=2.63) (Figure 3A).

Lipid Mediator Levels and Disease Severity

We next sought to determine whether a broad array of lipid mediators in nasal secretions were associated with asthma or sinonasal symptom severity. Total cysLTs did not differ by asthma severity. Among individual cysLTs, LTC_4 was most abundant. LTD_4 and LTE_4 levels were low but highest in the severe asthmatic group (Table 3). Asthma severity was inversely associated with levels of PGD_2 (p=0.02) and 9a,11b-PGF_{2a} (p=0.03). We examined potential correlations between each lipid mediator and sinonasal symptom burden, assessed via the 22-item sinonasal outcomes test (SNOT-22) at the time of

surgery (Table S2). LTE_4 levels in nasal secretions correlated with higher SNOT-22 scores (r=0.444, p=0.03) (Figure 3B), while a similar trend was observed for LTB_4 (r=0.330, p=0.11). SNOT-22 scores did not correlate with any other measured lipid mediator.

AERD Inflammatory Cluster Analysis

We used hierarchical cluster analysis incorporating the same seven cytokines to identify potential sub-endotypes of AERD. Analysis identified three potential AERD clusters based entirely on cytokine signatures (Figure 4). Visualization of the cluster structure showed discrete separation of each grouping and good cluster stability based on bootstrap validation (all clusters with stability ~ 0.7). The largest grouping (cluster 2, n=14) carried a type 2 high signature with elevated IL-4, IL-5, and IL-13 compared to the other clusters, as well as high levels of IL-6 (Figure S2). Cluster 1 (n=10) had low overall inflammatory burden, while cluster 3 (n=6) was type 2 low and type 1/type 3 high. Though somewhat limited by the small sample size in Cluster 3, no significant demographic differences were identified between clusters (Table 4). Patients in the type 2 high group (cluster 2) had the highest tissue eosinophil counts, worse SNOT-22 total and subdomain scores, and were more likely to have severe asthma, though none of these differences reached statistical significance.

Given the clearly defined role of arachidonic acid metabolism in AERD, we next compared levels of lipid mediators between the clusters. No differences in CysLTs were identified between the three clusters (Table 4). Conversely, the clusters varied significantly with respect to levels of 12,13 EpOME (p=0.003), 9-10-DiHOME (p=0.04), 13-HODE (p=0.02), 12-HETE (p=0.006), and TxB2 (p=0.02), with the highest levels in Cluster 1. There was a trend toward differences in 15-HETE (p=0.10), 20-HETE (p=0.08), PGD_2 (p=0.09) and its metabolite $PGF_{2\alpha}$ (p=0.10). Hierarchical cluster analysis was repeated with lipid mediators as input variables but resulted in heavily overlapping clusters that were comparatively unstable (data not shown).

DISCUSSION

Our data shows nasal cytokine dysregulation in AERD that is heterogeneous and potentially suggestive of disease sub-endotypes. We found that AERD could be discriminated from CRSwNP by elevations in IL-5, IL-6, IL-13, and IFN-γ. While comparatively few studies have specifically analyzed cytokine expression in patients with AERD discretely, many have reported characteristics similar to CRSwNP with a predominantly type 2 signature. However, recent studies that specifically examined patients with AERD as a distinct group, though limited by sample size, have reported conflicting and often surprising findings. Based on gene expression in nasal polyps, Steinke et al. reported reduced expression of the type 2 cytokines IL-5 and IL-13 in AERD (n=15) compared to CRSwNP, while IL-4 and IFN-γ were both increased[10]. They subsequently showed IFN-γ could promote the maturation of eosinophil progenitors and increase expression of genes involved in synthesis of cysLTs. A subsequent study by Stevens et al. found largely conflicting results[11]. AERD polyps (n=15) could be differentiated from CRSwNP by elevated protein levels of eosinophil cationic protein, but showed no differences in either IL-4, IL5, IL-13, or IFN-γ. Our study findings in a cohort double in size help to reconcile these prior reports and suggests that conflicting results may be indicative of heterogeneity within the AERD population.

Collectively, our data suggests that AERD is characterized by a predominantly type 2 signature, but also has characteristics of both type 1 and type 3 inflammation. Our finding that IL-6 is elevated in AERD and is highest in the cluster with the highest levels of type 2 cytokines may explain the propensity towards severe clinical presentations in AERD and a source for resistance to type 2 targeted therapies. Alterations in the IL-6 pathway have previously been reported in CRSwNP[24]. In asthma, elevated plasma IL-6 is associated with low lung function and increased exacerbations[25], neutrophilic and mixed granulocytic airway inflammation[26],and is implicated in promoting the conversion of iTreg cells into TH17-like cells[27]. Our group recently reported that nasal mucus IL-6 levels are highest in patients with

mixed granulocytic sinus infiltrate and more severe disease[15]. The elevations in IL=17A and INF-γ similarly highlight the complex inflammatory milieu of AERD.

We sought to deconvolute this inflammatory heterogeneity using an unstructured statistical approach and found that patients with AERD could be differentiated into 3 potential inflammatory clusters. Approximately half of all patients fell into a cluster with very high type 2 cytokines, with the remainder instead characterized by low inflammatory burden or elevated type 1 and type 3 cytokines. To our knowledge, this is the first study to find potential sub-endotypes of AERD using inflammatory mediators alone. Using latent class analysis, Bochenek et al. identified four subphenotypes of AERD using clinical characteristics and a small number of serum and urine biomarkers[28]. Though only a single lipid mediator was measured (urinary LTE_4), the results did show that the class with the highest LTE_4 levels also had the greatest upper respiratory and/or sinus symptoms. This is consistent with our study, which identified LTE_4 as the only lipid mediator that correlated with worse SNOT-22 scores and was significantly elevated in AERD only. A similar approach was subsequently used by Celejewska-Wojcik et al., this time incorporating 16 variables that included three select eicosanoids (PGD_2, PGE_2, LTE_4) in induced sputum supernatants and resulting in three distinct subphenotypes[8]. This study found that the class with the highest levels of pro- and anti-inflammatory arachidonic acid metabolites was characterized by relatively well-controlled mild-to-moderate asthma and mixed eosinophilic and neutrophilic infiltrate in induced sputum. However, neither of these studies measured cytokine levels and were therefore unable to link clinical characteristics and/or eicosanoid levels with inflammatory signatures.

The results of the current study suggest that patients with AERD are fairly homogenous with respect to lipid mediators in nasal secretions, though individual mediators clearly have well-defined physiological roles and may also act as disease modifiers. Patients with mild asthma severity had a global increase in cyclooxygenase metabolites with elevated levels of PGD_2 and 9a,11b-PGF_{2a}, both CRTH2 agonists important in effector cell chemotaxis to the tissue[29]. Other PGD_2 metabolites which were not assessed in this study and have been reported to have both pro- and anti-inflammatory actions may explain the differences observed by asthma severity[30]. Similarly, the anti-inflammatory PGE_2 was highest,

but not significantly elevated, in the mild asthma severity subgroup. The balance between pro-inflammatory and anti-inflammatory lipid mediators may dictate clinical severity as has been suggested by mediator analyses in the lower airways[8]. We also found that sinonasal symptoms correlated with mucus levels of LTE_4, but not with any other measured lipid mediator. LTE_4 is a key mediator of chemosensory cell number and function[31], epithelial cell mucin production[32], and mast cell activation[33] in the respiratory tract. It is notable that patients with AERD had a dramatic elevation in mucus LTE_4 levels compared with controls and CRSwNP despite similar levels of LTC_4. Local differences in the enzymes required for LTC_4 conversion to LTD_4 (gamma-glutamyl leukotrienase and gamma-gluatmyl transpeptidase) and LTE_4 (dipeptidase) or ω-oxidation which would render LTE_4 undetectable in our MS methods may explain the differences observed between clinical phenotypes[34-37]. However, the variability of most lipid mediators among patients with AERD was comparatively low, suggesting that heterogeneity in these patients may be driven by other mediators. As shown here, hierarchical cluster analysis using type 1, type 2, and type 3 cytokines resulted in well-delineated and stable disease clusters, while a similar approach using physiologically relevant eicosanoids was not able to effectively discriminate patients in any meaningful way.

We measured what to our knowledge is the largest array of nasal lipid mediators in patients with AERD. We found the highest levels of pro- and anti-inflammatory mediators in cytokine cluster 1, which was generally associated with lower cytokine inflammatory burden and moderate disease severity. Cluster 1 (cytokine low) demonstrated the highest amount of LA derivatives 12,13-EpOME, 9-10-DiHOME, and 13-HODE, and eicosanoids 12-HETE and TxB_2. This combination of LA and AA-derived lipid mediators may come from neutrophil (12,13-EpOME (isoleukotoxin)[38], 9,10-DiHOME (leukotoxin diol)[39], 13-HODE[40]) and platelet (TxB_2, 12-HETE[41]) sources. 12,13-EpHOME, generated by CYP450 from LA during oxidative burst, uncouples mitochondrial respiration[42] and is present in the lavage fluid of acute respiratory distress syndrome[43]. 9,10-diHOME is generated from 9(10)-EpOME (leukotoxin) by soluble epoxide hydrolase in neutrophils[39]. 13-HODE is produced by non-enzymatic lipid peroxidation of LA. In a human endothelial cell line, 9,10-DiHOME, 13-HODE, and 12-HETE were generated in response to

lipopolysaccharide stimulation and suppressed TNF-α[44]. In epithelial cells, 15-lipooxygenase (LO) expression is induced by IL-13 and contributes to the generation of 15-HETE from AA and 13-HODE from LA[45]. 13-HODE activates TRPV1, a neurosensory receptor, which is increased in CRSwNP and triggers IgE-independent mast cell activation[46]. Platelet-derived 12-HETE is a potent mediator of neutrophil chemotaxis[47]. Depending on the stimulus studied, 12-HETE has been shown to have promoting and suppressing effects on platelet activation[48]. Notably 12-HETE interacts with the TXA_2 receptor (TP) and inhibits TXA_2-induced platelet aggregation[49, 50]. Platelets and TP have been identified as key factors required for inflammation in AERD[51, 52]. Platelets serve as a source of the alarmin IL-33[53] and the excessive cysLT generation characteristic of AERD[54, 55]. In animal models of AERD, inhibition of TP blocks the acute aspirin-induced respiratory reaction, cysLT generation and mast cell activation[52]. Together these data support that AA and LA derivates outside of the classical COX and 5-LO pathways may be key regulators of nasal inflammation in a subgroup of patients with AERD.

Some limitations to the current study deserve discussion and may limit the generalizability of our findings. Patients were seen at a single tertiary referral center within the southeastern United States and as such may not be representative of patients with AERD from other geographic regions. The sample size of patients with AERD, though substantially larger than similar recent studies[10, 11], was likely insufficiently powered for some comparisons and limited the number of variables that could be incorporated into cluster analyses. Additionally, 63% of AERD patients were being treated with a leukotriene receptor antagonist at the time of sample collection, which could potentially affect levels of some lipid mediators (though none were taking leukotriene synthesis inhibitors). We also measured cytokines and lipid mediators in sinonasal mucus rather than in tissue, as had been performed in a small number of other studies[10, 11]. However, we have found a strong correlation between levels of mediators in mucus and tissue (Fig. S3). Nonetheless, our findings suggest that patients with AERD that are phenotypically similar may have substantial differences in underlying inflammatory burden. Though AERD has a near universal association with type 2 inflammation, we additionally found that type 1 and type 3 cytokine mediators may have pathophysiological roles in many patients, a finding that may have important implications for

treatment. Humanized monoclonal antibodies that target type 2 inflammation cytokine pathways are now recognized as effective therapeutics for CRSwNP, with some studies showing a potential subgroup effect in patients with AERD.[44] This may be secondary to higher levels of type 2 cytokines in patients with AERD, however, the inflammatory heterogeneity shown here suggests that these therapeutics may have reduced efficacy in a substantial subset of patients.

CONCLUSION

Patients with AERD display heterogeneous inflammatory burden with variable levels of type 1, type 2, and type 3 cytokines. Between patient differences in CysLTs and other lipid mediators were limited but select mediators were associated with both asthma and sinonasal symptom severity. Our findings suggest that sub-endotypes of AERD may exist and point to the need for further research into AERD pathophysiology. Improved characterization of disease subtypes or endotypes may help to effectively identify patients most likely to benefit from current and future targeted therapies.

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TABLE LEGENDS

Table 1. Demographic and clinical characteristics of study population. Values are presented as means +/- SDs or medians with interquartile ranges, depending on the normalcy of the data. Differences between groups were assessed by using the Kruskal-Wallis test or X^2 analysis. Boldface text indicates a P value of less than 0.05. AERD, Aspirin-exacerbated respiratory disease; BMI, body mass index; NCS, nasal corticosteroid; LTR, leukotriene modifier; SNOT-22, 22-item sinonasal outcomes test.

Table 2. Lipid mediator levels in control, CRSwNP, and AERD patients. Mucus levels of lipid mediators are shown for each group. All mediator levels are presented as ng/mL. Significant differences between groups were identified by the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Data are presented as medians with interquartile range. Boldface text indicates P value less than 0.05. *, $p<0.05$ compared to controls; #, $p<0.05$ compared to CRSwNP.

Table 3. Mucus levels of lipid mediators in patients with AERD based on asthma severity. Median levels of lipid mediators in patients with AERD and mild, moderate, or severe asthma are shown. All mediator levels are presented as ng/mL. Significant differences between groups were identified by the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Data are presented as medians with interquartile range. Boldface text indicates P value of less than 0.05.

Table 4. Demographic, clinical characteristics, and nasal mucus lipid mediators of AERD inflammatory clusters. Values are presented as means +/- SDs or medians with interquartile ranges, depending on the normalcy of the data. All mediator levels are presented as ng/mL. Data are presented as medians with interquartile range. Differences between groups were assessed by using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons or X^2 analysis. Boldface text indicates a P value of

604 less than 0.05. AERD, Aspirin-exacerbated respiratory disease; BMI, body mass index; NCS, nasal
605 corticosteroid; LTR, leukotriene modifier; SNOT-22, 22-item sinonasal outcomes test.
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FIGURE LEGENDS

Figure 1. Mucus cytokines in AERD, CRSwNP and non-CRS control patients. Cytokine values are plotted on a log scale. Solid lines indicate medians with interquartile ranges.

Figure 2. Mucus lipid mediators in AERD, CRSwNP, and non-CRS control patients. Solid lines indicate medians with interquartile range.

Figure 3. Variance comparison for cytokines and lipid mediators. Log-normalized and centered raw cytokine and lipid mediator values are visualized using boxplots for a graphical comparison of the relative variability of each biomarker. Values are expressed as the median with interquartile range. Omnibus testing between biomarkers using the coefficient of variation showed statistically significant differences ($p < 0.001$) between groups. Note the relatively lower variance in lipid mediators compared to cytokines.

Figure 4. Identification of inflammatory disease clusters in patients with AERD. A) Dendogram representing hierarchical cluster analysis of patients with AERD. Hierarchical cluster analysis was performed by using the Ward method on squared Euclidian distances, with seven cytokines as biological variables. B) PCA plot showing patient clusters based on their similarity.

650 **Table 1.**

	Control	CRSwNP	AERD	p value (CRSwNP vs AERD)
Number	64	109	30	
Age, years	51.6 +/- 15.4	50.4 +/- 15.2	47.8 +/- 11.5	0.31
Sex, no. female (%)	40 (68)	35 (32)	12 (40)	0.51
Asthma, no. (%)	1 (2)	37 (33.9)	30 (100)	**<0.001**
Allergic rhinitis, no. (%)	5 (8)	64 (58.7)	23 (76.7)	0.27
Prior surgery, no. (%)	0 (0)	40 (36.7)	21 (70)	**0.02**
BMI (kg/m2)	33.1 ± 9.1	29.1 ± 5.9	29.8 ± 4.7	0.65
NCS, no. (%)	1 (2)	90 (82.6)	23 (76.7)	0.75
LTR, no. (%)	3 (5)	26 (23.9)	19 (63.3)	**<0.001**
current smoker, no. (%)	1 (2)	5 (4.6)	0 (0)	0.24
SNOT-22 score		42.1 +/- 21.1	50.7 +/- 21.6	0.08
Rhinologic		12.3 +/- 5.7	15.0 +/- 5.5	**0.04**
Extranasal		7.2 +/- 3.5	9.2 +/- 3.2	**0.01**
Ear/Facial		7.0 +/- 5.3	9.3 +/- 5.5	0.08
Psychological		11.5 +/- 8.9	12.7 +/- 8.0	0.56
Sleep		10.3 +/- 7.1	10.8 +/- 7.9	0.77
CT score		15.9 ± 4.2	20.3 ± 3.5	**<0.001**

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653 **Table 2.**

Mediator	Control (n=33)	CRSwNP (n=34)	AERD (n=30)	p value
12,13-EpOME	11359 (7953-19637)	8591 (4846-14877)	5782 (2737-12313)*	**0.005**
9,10-DiHOME	15.8 (9.4-26.6)	11.4 (8.9-11.4)	5.4 (2.7-23.4)*#	**0.003**
12,13-DiHOME	23.4 (14.4-41.3)	27.6 (19.9-40.7)	1.5 (0.01-4.1)*#	**<0.001**
9,10-EpOME	1150 (687.9-3835)	652 (394.5-929.7)*	249 (156-450)*#	**<0.001**
13-HODE	9804 (6807-16566)	7335 (4228-12482)	8852 (4163-16605)	0.17
8-HETE	86.0 (68.6-136.7)	55.0 (36.8-103.6)	29.0 (14.8-64.0)*#	**<0.001**
15-HETE	8259 (5359-10414)	6554 (4050-9011)	3496 (1795-6715)*	**0.006**
12-HETE	921.7 (658.1-1210)	676.5 (406.4-945.0)	474.2 (216.9-595.6)*#	**<0.001**
11,12-EET	1295 (845-5892)	856.5 (587.5-1289)	228.3 (164.1-336.4)*#	**<0.001**
14,15-EET	1688 (1115-2583)	1267 (697-1629)	725 (467-981)*#	**<0.001**
20-HETE	181126 (99675-253998)	152415 (82825-188409)	61850 (36426-117669)*#	**<0.001**
TxB_2	4.9 (1.4-16.5)	6.3 (1.2-15.6)	16.9 (6.2-47.9)*#	**0.003**
PGD_2	6.3 (4.0-13.3)	21.1 (11.1-41.6)*	28.1 (15.0-63.8)*	**<0.001**
15-keto-PGE_2	0.77 (0.41-1.30)	0.97 (0.39-1.53)	1.36 (0.67-1.82)*	**0.03**
PGE_2	105.0 (68.1-137.6)	73.8 (56.3-149.9)	98.4 (49.5-149.8)	0.92
PGF_{2a}	18.4 (12.9-35.7)	15.7 (9.6-20.5)	9.5 (0.01-16.8)*	**0.001**
9a,11b-PGF_{2a}	1.3 (0.3-3.7)	3.0 (1.7-9.8)	9.7 (0.01-20.9)*	**0.005**
LTB_4	12.7 (9.3-22.1)	11.1 (5.6-24.3)	0.01 (0.01-11.3)*#	**<0.001**
LTC_4	31.9 (13.8-59.2)	53.0 (27.6-80.0)	32.0 (14.9-72.0)	0.16
LTD_4	0.01 (0.01-0.34)	0.23 (0.01-1.14)	1.51 (0.01-2.65)*	**<0.001**
LTE_4	0.17 (0.03-0.40)	0.62 (0.16-2.78)*	4.97 (1.87-12.75)*#	**<0.001**
Tot cysLTs	33.0 (16.9-60.4)	55.7 (34.3-87.1)	43.9 (21.3-79.2)	0.10

654 **Table 3.**

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Mediator	Mild Asthma (n=3)	Moderate Asthma (n=5)	Severe Asthma (n=22)	p value
12,13-EpOME	4202 (2426-20393)	7500 (2116-14472)	5782 (2737-12313)	0.90
9,10-DiHOME	4.38 (4.00-23.60)	2.70 (0.75-20.61)	6.05 (3.31-23.36)	0.74
12,13-DiHOME	1.88 (0.01-4.08)	0.89 (0.01-3.73)	1.53 (0.01-4.32)	0.91
9,10-EpOME	315.8 (151.3-534.7)	191.0 (125.3-570.8)	274.9 (156.4-450.0)	0.90
13-HODE	9155 (5108-29380)	11236 (2724-19669)	8461 (4070-16605)	0.75
8-HETE	16.0 (13.5-61.2)	29.0 (15.5-48.4)	29.3 (14.8-72.5)	0.71
15-HETE	3496 (2159-6715)	3023 (1465-9557)	3594 (1790-6717)	0.98
12-HETE	376 (340-596)	534 (146-679)	474 (217-643)	0.99
11,12-EET	220.9 (164.6-336.6)	274.8 (168.8-661.1)	223.5 (159.4-337.9)	0.81
14,15-EET	642.9 (458.4-969.5)	1079 (381.9-1739)	724.7 (466.6-959.2)	0.59
20-HETE	49723 (37749-131101)	42732 (28616-150425)	67307 (35685-116309)	0.95
TxB_2	20.9 (10.6-46.1)	12.9 (6.8-31.1)	16.9 (5.8-81.7)	0.83
PGD_2	154.3 (78.1-161.9)	17.9 (6.9-36.5)	27.3 (15.0-60.2)	**0.02**
15-keto-PGE_2	2.35 (2.06-2.81)	0.67 (0.44-1.59)	1.31 (0.78-1.58)	0.10
PGE_2	149.8 (98.4-155.4)	73.3 (41.0-191.1)	90.2 (46.8-143.1)	0.52
PGF_{2a}	0.01 (0.01-46.7)	14.0 (4.76-22.1)	7.6 (0.01-15.2)	0.71
9a,11b-PGF_{2a}	35.6 (34.3-40.4)	13.9 (0.01-18.0)	8.3 (0.01-20.2)	**0.03**
LTB_4	0.01 (0.01-81.2)	3.9 (0.01-93.6)	0.01 (-0.01-10.7)	0.54
LTC_4	125.7 (32.0-470.6)	38.7 (13.0-120.7)	22.9 (6.23-64.77)	0.10
LTD_4	0.01 (0.01-0.01)	0.01 (0.01-2.54)	2.06 (0.01-4.04)	**0.05**
LTE_4	1.29 (0.84-1.87)	3.73 (1.82-17.3)	5.34 (3.67-12.75)	0.15
Total cysLTs	126.6 (33.3-472.2)	43.9 (32.1-122.5)	36.6 (17.7-74.1)	0.16

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658 **Table 4.**

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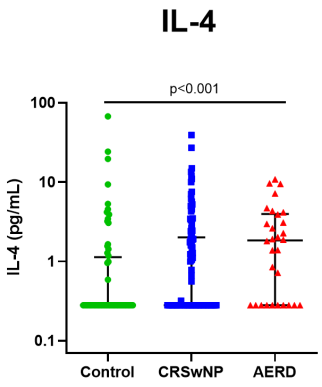
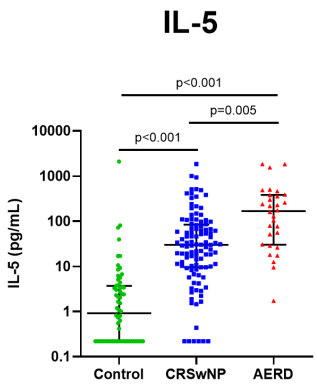
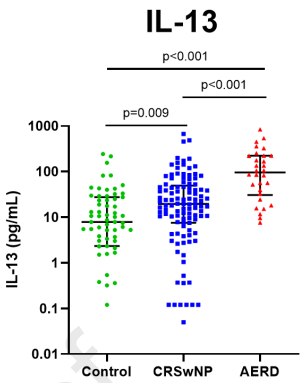
	Cluster 1	Cluster 2	Cluster 3	p value
No.	10	14	6	
Age (years)	41.8 +/- 12.4	50.0 +/- 12.4	47.8 +/- 14.4	0.37
Sex, no. (% female)	5 (50)	6 (43)	1 (17)	0.40
Race, no. (% white)	10 (100)	10 (71)	6 (100)	0.26
BMI (kg/m2)	29.1 +/- 7.0	30.6 +/- 3.2	29.3 +/- 4.1	0.73
Allergic Rhinitis, no. (%)	9 (90)	9 (64)	5 (83)	0.31
NCS, no. (%)	7 (70)	11 (79)	5 (83)	0.81
LTR, no. (%)	6 (60)	10 (71)	3 (50)	0.64
Prior surgery, no. (%)	6 (60)	11 (79)	4 (67)	0.61
# of prior surgeries	1.0 (0.0-1.8)	1.0 (1.0-2.8)	2.0 (0.5-4.3)	0.48
mean Eos/HPF	124 (96-257)	157 (80-250)	108 (95-120)	0.59
SNOT-22 score	46.6 +/- 24.6	56.7 +/- 21.5	42.3 +/- 18.0	0.31
Rhinologic	11.7 +/- 4.9	16.8 +/- 5.8	15.5 +/- 4.5	0.15
Extranasal	8.7 +/- 4.5	9.9 +/- 2.5	8.5 +/- 3.0	0.47
Ear/Facial	7.6 +/- 5.6	11.9 +/- 5.4	6.3 +/- 3.2	0.08
Psychological	13.0 +/- 8.2	14.6 +/- 8.4	9.0 +/- 7.0	0.41
Sleep	12.3 +/- 9.4	11.4 +/- 6.4	8.2 +/- 9.2	0.63
CT score	21.1 +/- 2.9	20.5 +/- 2.6	19.3 +/- 3.9	0.53
Asthma Classification				0.74
Persistent	9 (90)	13 (93)	6 (100)	
Intermittent	1 (10)	1 (7)	0 (0)	
Asthma Status				0.61
Mild	2 (20)	1 (7)	0 (0)	
Moderate	2 (20)	1 (7)	2 (33)	
Severe	7 (70)	12 (86)	4 (67)	
12,13-EpOME	11304 (6495-14686)	4065 (2224-10679)	2737 (747-10859)	**0.03**
9,10-DiHOME	22.7 (8.6-25.8)	4.7 (3.2-14.4)	3.4 (2.0-4.7)	**0.04**
12,13-DiHOME	1.2 (0.2-3.7)	1.7 (0.0-3.7)	0.0 (0.0-2.2)	0.48
9,10-EpOME	402 (280-4920)	235 (131-401)	156 (61-530)	0.07
13-HODE	15896 (11297-19632)	5283 (3880-15353)	4767 (1803-7366)	**0.02**
8-HETE	38.0 (18.4-69.7)	25.0 (20.1-41.6)	51.5 (18.4-64.4)	0.61
12-HETE	590 (545-931)	321 (205-516)	283 (193-486)	**0.006**
15-HETE	5790 (3141-6848)	4569 (2508-7080)	1788 (1297-3143)	0.10
11,12-EET	211 (166-272)	237 (168-392)	224 (155-270)	0.76
14,15-EET	882 (657-1144)	794 (532-972)	456 (188-740)	0.25
20-HETE	102474 (45493-125811)	84706 (39573-128811)	32647 (23116-59291)	0.08
TxB_2	63.8 (19.3-90.5)	13.4 (4.7-19.3)	17.8 (8.4-27.7)	**0.03**
PGD_2	44.2 (27.6-70.2)	25.2 (11.4-67.4)	12.5 (3.6-24.5)	0.08
15-keto-PGE_2	1.3 (0.9-1.6)	1.4 (0.5-1.8)	1.1 (0.6-1.5)	0.99

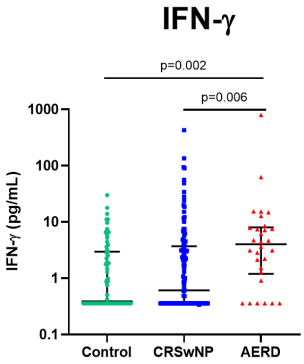
	PGE_2	108.5 (75.8-155.8)	96.7 (70.1-137.8)	37.3 (23.1-113.6)	0.30
	PGF_{2a}	23.1 (7.5-33.6)	3.0 (0.0-12.8)	10.8 (1.9-14.9)	0.10
	9a,11b-PGF_{2a}	14.9 (8.5-21.5)	9.0 (0.0-20.9)	6.2 (0.0-13.4)	0.30
	LTB_4	0.0 (0.0-11.2)	1.9 (0.0-11.2)	0.0 (0.0-4.7)	0.90
	LTC_4	47.7 (19.7-74.7)	24.6 (2.8-55.5)	30.2 (19.0-38.4)	0.46
	LTD_4	2.8 (0.2-4.1)	1.2 (0.0-2.1)	1.8 (0.4-2.3)	0.53
	LTE_4	4.4 (3.7-10.5)	9.8 (4.6-13.4)	4.5 (0.9-9.9)	0.52
	Total cysLTs	65.7 (32.0-82.6)	29.0 (18.2-74.0)	40.3 (19.4-76.7)	0.41

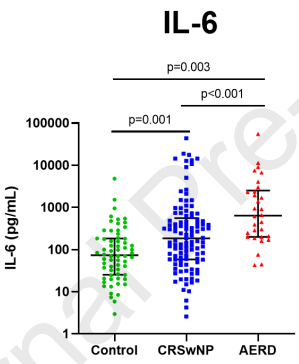
660

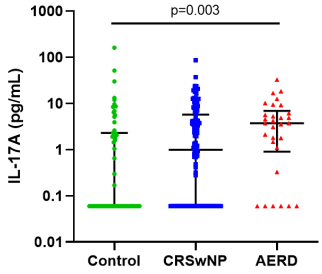


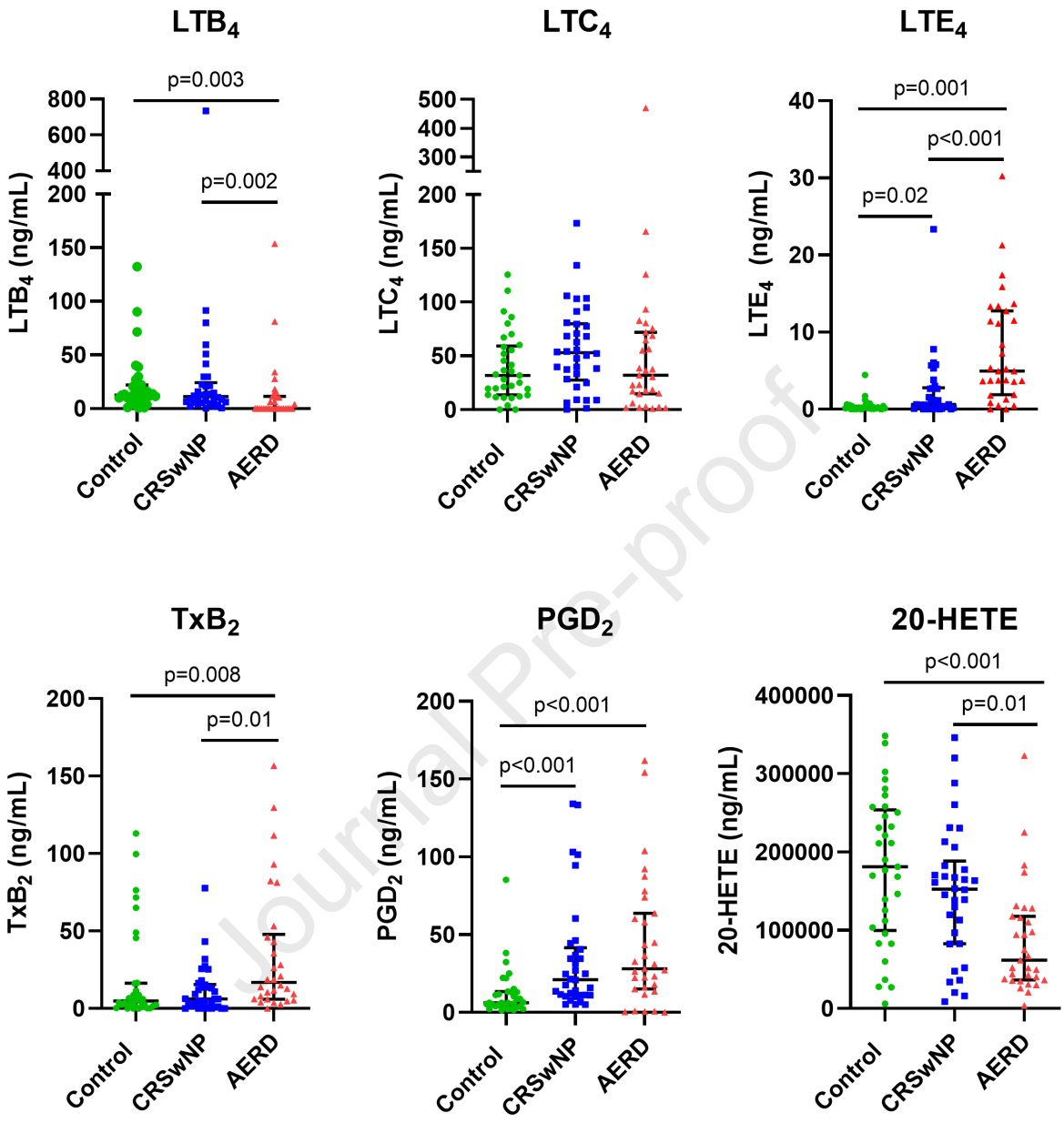












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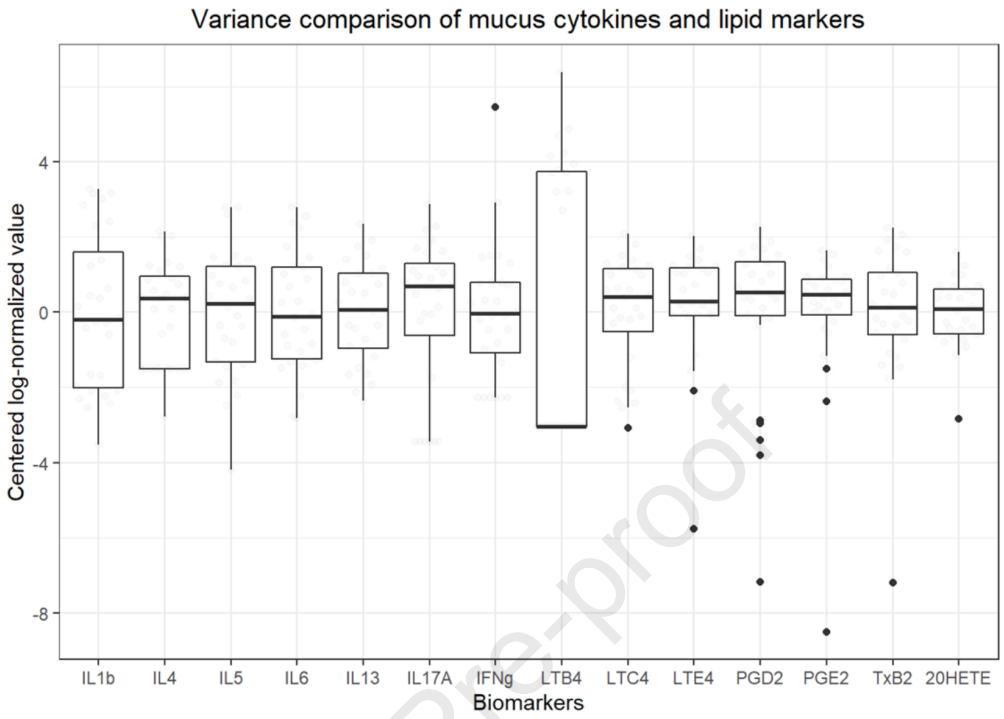

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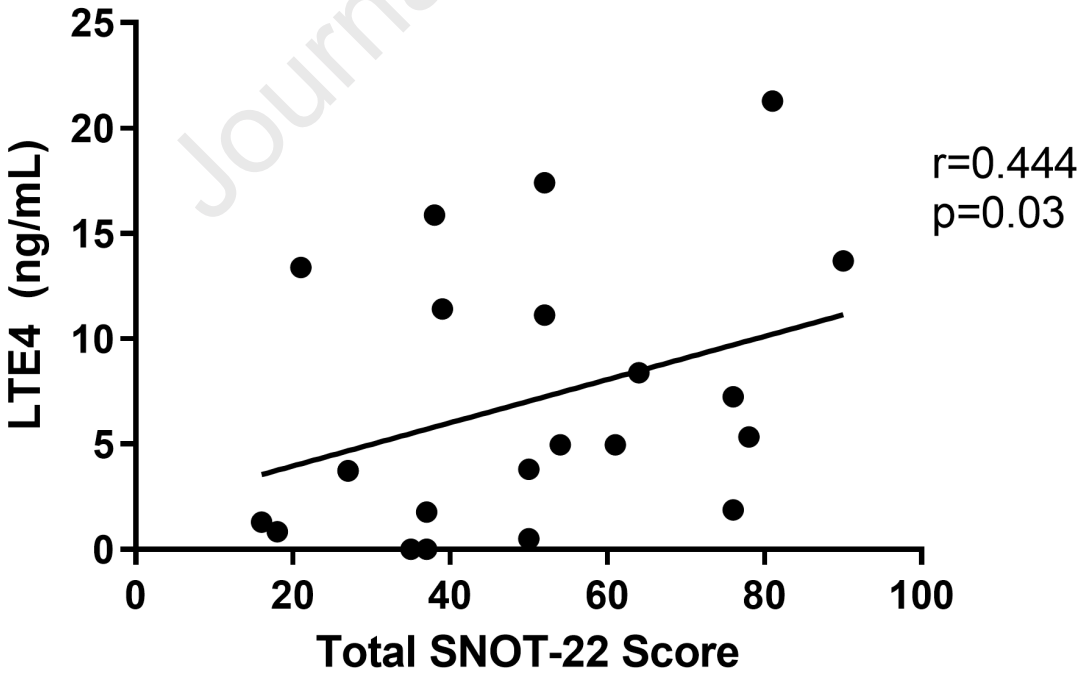

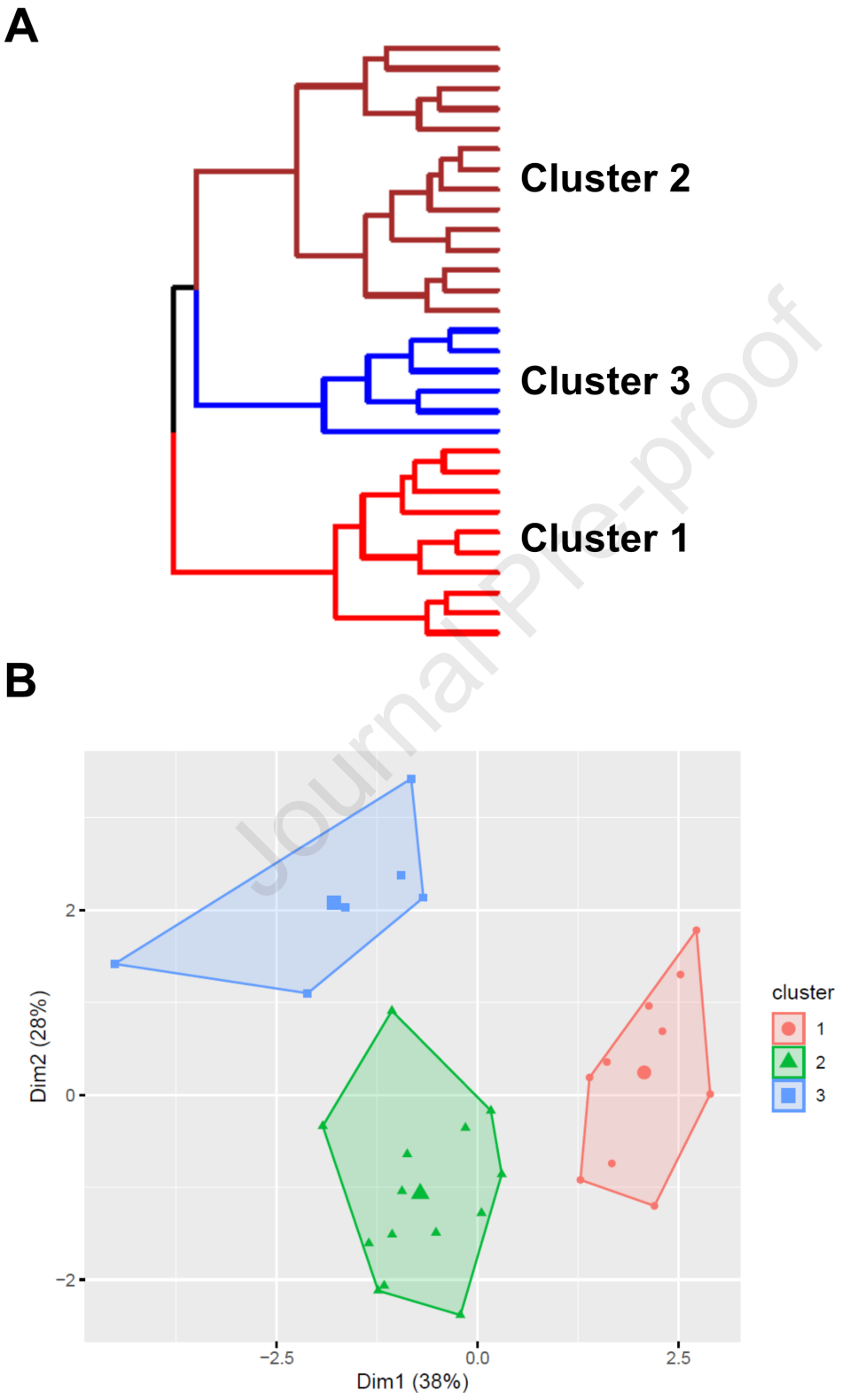

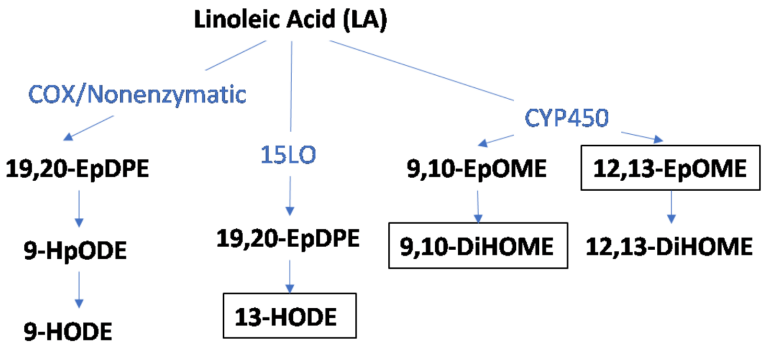

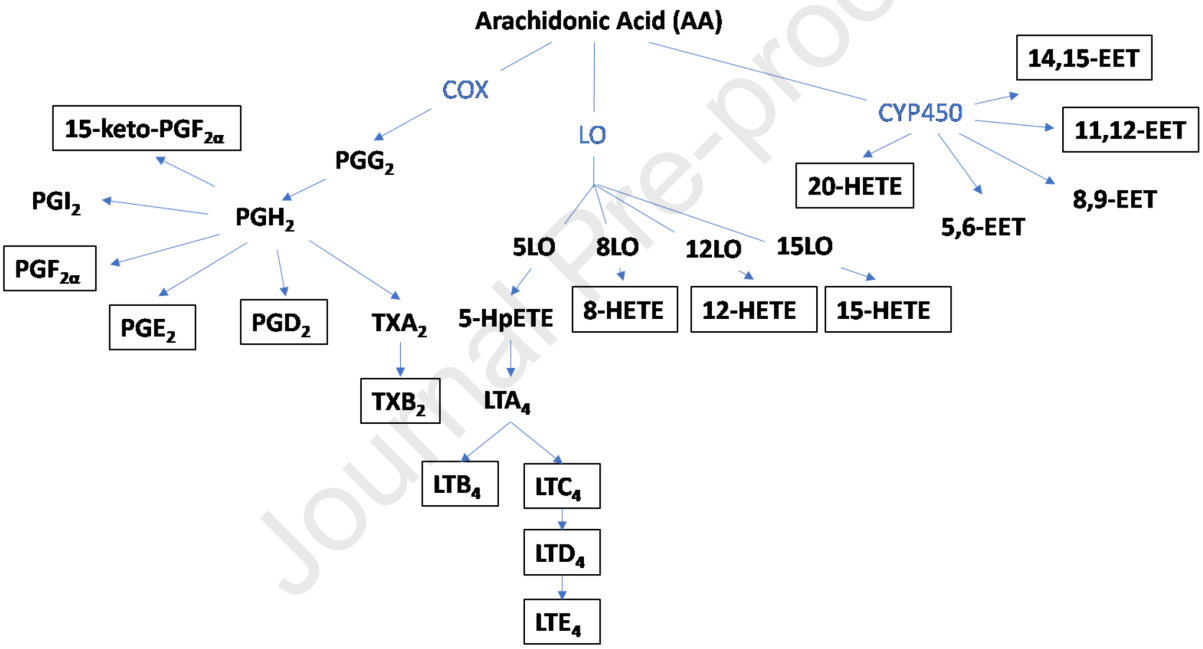

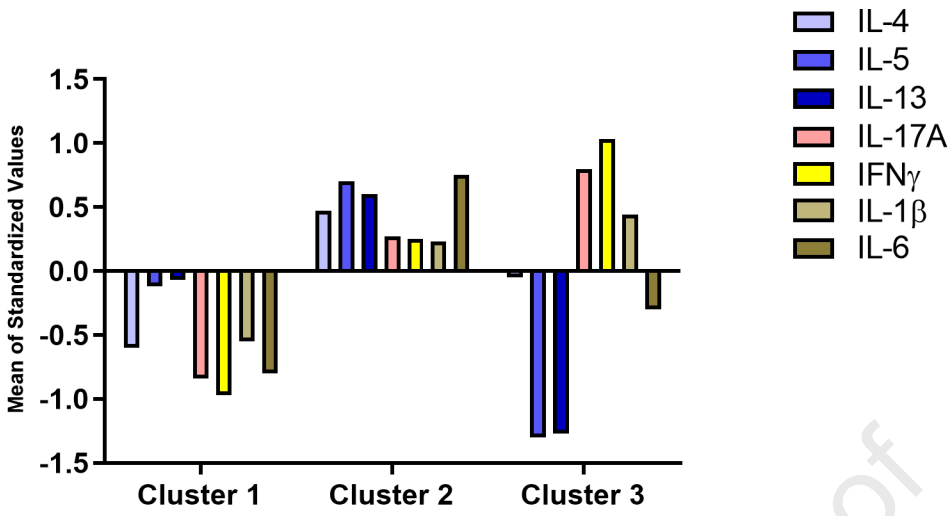

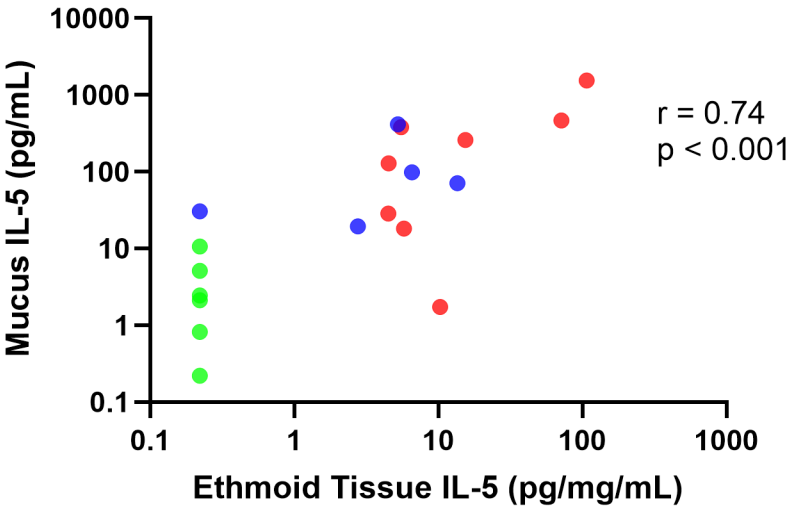

Figure Legends

Figure S1. Linoleic Acid (LA) and Arachidonic Acid (AA) metabolism. Lipid mediators evaluated by (UPLC)-mass spectrometry are highlighted in boxes.

Figure S2. Cytokine signature in AERD inflammatory clusters. The mean standardized levels of representative cytokines representing type 1 (IFN-γ), Type 2 (IL-4, IL-5, IL-13), type 3 (IL-17A), and innate immune (IL-1β, IL-6) signatures are shown for each disease cluster.

Figure S3. Correlation of IL-5 levels in mucus and ethmoid tissue. Green, control (n=7); Blue, CRSwNP (n=5); Red, AERD (n=8). R value calculated via Spearman correlation.

Table Legends

Table S1. Mucus cytokine levels in AERD, CRSwNP, and non-CRS control patients. Median cytokine levels of control subjects and patients with CRSwNP and AERD are shown for seven cytokines. All cytokine values are presented as pg/mL. Significant differences between groups were identified by the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Data are presented as medians with interquartile range. Boldface text indicates P value of less than 0.05. *, $p<0.05$ compared to control; #, $p<0.05$ compared to CRSwNP.

Table S2. SNOT-22 score correlation with lipid mediator levels in subjects with AERD. The correlation of lipid mediator levels with total SNOT-22 score was evaluated using Spearman correlation. Boldface indicates a P value less than 0.05. SNOT-22, 22-item sinonasal outcomes test.

Table S1.

	Control (n=64)	CRSwNP (n=109)	AERD (n=30)	p value
IL-1β	84.8 (30.1-300.7)	128.1 (23.1-521.1)	108.0 (17.7-1097.2)	0.67
IL-4	0.28 (0.28-1.04)	0.28 (0.28-1.92)*	1.85 (0.28-3.70)*	**<0.001**
IL-5	0.92 (0.22-3.56)	30.2 (9.6-82.1)*	168.0 (46.3-374.1)* #	**<0.001**
IL-6	73.4 (25.9-181.4)	184.5 (58.9-521.5)*	636 (206-2346)* #	**<0.001**
IL-13	10.4 (3.7-28.6)	19.5 (7.7-46.0)	95.7 (37.7-222.7)* #	**<0.001**
IL-17A	0.06 (0.06-2.25)	0.99 (0.06-5.11)	3.75 (1.39-6.05)*	**0.004**
IFN-γ	0.39 (0.36-2.90)	0.61 (0.36-3.50)	4.02 (1.46-7.79)* #	**0.002**

Table S2. SNOT-22 score correlation with lipid mediator levels in subjects with AERD

Mediator	Spearman r	p value
12,13-EpOME	-0.003	0.99
9,10-DiHOME	0.201	0.35
12,13-DiHOME	0.159	0.46
9,10-EpOME	0.130	0.55
13-HODE	0.054	0.80
8-HETE	0.018	0.93
12-HETE	-0.014	0.95
15-HETE	0.000	0.99
11,12-EET	-0.130	0.54
14,15-EET	-0.053	0.81
20-HETE	-0.060	0.78
TxB_2	0.087	0.69
PGD_2	0.017	0.94
15-keto-PGE_2	-0.139	0.52
PGE_2	-0.070	0.75
PGF_{2a}	0.023	0.92
9a,11b-PGF_{2a}	-0.181	0.40
LTB_4	0.330	0.11
LTC_4	0.018	0.93
LTD_4	0.174	0.42
LTE_4	0.444	**0.03**