

# Severe Type 2 Inflammation Leads to High PAF-Associated Pathology in Chronic Rhinosinusitis with Nasal Polyps (CRSwNP): Hierarchical Cluster Analysis Using Bulk RNA Barcoding and Sequencing

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## Article

# Severe Type 2 Inflammation Leads to High PAF-Associated Pathology in Chronic Rhinosinusitis with Nasal Polyps (CRSwNP): Hierarchical Cluster Analysis Using Bulk RNA Barcoding and Sequencing

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**Abstract:** Platelet-activating factor (PAF) is a phospholipid-derived inflammatory mediator, triggering various inflammatory conditions, including eosinophil activation and recruitment. Despite the unclear mechanism underlying the formation and maintenance of nasal polyp in chronic rhinosinusitis with nasal polyps (CRSwNP), there is strongly indication of its association with type 2 inflammation. This study aimed to quantify PAF-associated gene expression, examining enzymes involved in PAF synthesis (lysophosphatidylcholine acyltransferase 1 (*LPCAT1*), *LPCAT2*, *LPCAT3*, and *LPCAT4*), and PAF degradation (PAF acetylhydrolases 1 B2 (*PAFAH1B2*), *PAFAH1B3*, and PAF acetylhydrolases 2 (*PAFAH2*)), and PAF receptor (*PTAFR*). Transcriptomic analysis encompassed CRSwNP, including eosinophilic CRS (ECRS) (n=9), nonECRS (n=8), and a subgroup of ECRS with aspirin exacerbated respiratory disease (Asp) (n=3) based on the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) criteria. Gene expression profiles from nasal polyps in CRSwNP patients and the uncinate process in normal subjects (controls) (n=6) were analyzed using bulk RNA barcoding and sequencing (BRB-seq). Hierarchical analysis, utilizing the average linkage method with iDEP1.1, clarified the relationship between Differentially Expressed Genes (DEG) expressions and cluster formation. Two clusters (cluster 1 (n=11) and cluster 2 (n=9)) of CRSwNP divided by hierarchical analysis showed distinct gene expression profiles in the specific pathway of “cytokine-cytokine receptor interaction” in KEGG pathway and upregulated type 2 inflammatory gene expression in cluster 2. In CRSwNP subclassification of ECRS, nonECRS, and Ctrl, *PTAFR* was only upregulated in ECRS and nonECRS. In cluster analysis, cluster 1 showed significant downregulation of *LPCAT2* and upregulation of *PTAFR* expression, while cluster 2 showed significant upregulation of *LPCAT1*, *PAFAH1B2*, and *PTAFR* and downregulation of *PAFAH2* expression. These findings suggest that cluster 2, characterized by severe type 2 inflammatory gene expression, manifest a prominent PAF-associated pathophysiology. Understanding this high PAF-associated pathophysiology in cluster 2 could provide valuable insights into the treatment and management of CRSwNP.

**Keywords:** paranasal sinuses; chronic rhinosinusitis (CRS); BRB-seq; nasal polyps; Differentially Expressed Genes (DEG) analysis; hierarchical analysis; pathway analysis; Type 2 inflammation; *LPCAT1*; *LPCAT2*; *PAFAH1B2*; *PAFAH1B3*; *PAFAH2*; *PTAFR*

## 1. Introduction

Chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is a complex inflammatory disorder characterized by diverse clinical presentations and underlying inflammatory profiles. It can be categorized into eosinophilic CRS (ECRS) and non-ECRS based on the extent of eosinophilic infiltration in sinonasal tissue [1]. Pathological association between the inflammatory endotype of type 1, 2, and 3 and phenotypes of CRSwNP are deeply associated with the pathophysiology of CRSwNP [2]. ECRS, a subtype characterized by Th2-dominant inflammation, often results in less favorable surgical outcomes [1,3], and cases of ECRS with aspirin exacerbated respiratory disease (AERD) mostly show more severe surgical outcome due to the severe type 2 inflammation [4].

The precise mechanism behind the formation and maintenance of nasal polyps in CRSwNP remains unclear, but there's strong evidence suggesting an association with type 2 inflammation. The pathophysiology of ECRS, especially in ECRS with AERD, involves the dysregulation in the synthesis of glycerophospholipid mediators including leukotrienes, prostaglandin, thromboxane, and *PAF* [5]. In the context of type 2 inflammatory reactions, IgE-mediated hypersensitivity triggers immune cells to process allergens, prompting a B-cell mediated immune response. This leads to the cross-linking of IgE antibodies on inflammatory cells, initiating mast cell degranulation and the release of various mediators like *PAF*, histamine, and pro-inflammatory cytokines [6,7]. These mediators induce both early and late-phase allergic responses, resulting in cellular inflammation by recruiting eosinophils, basophils, monocytes, and lymphocytes. *PAF*, a phospholipid-derived inflammatory mediator released by various cell types [8], plays a pivotal role in multiple inflammatory states, recruiting eosinophils and neutrophils, triggering leukocyte degranulation and adhesion, and generating free radicals like superoxide and hydroxyl anions in the nasal mucosa [9]. A previous report mentioned that a large amount of *PAF* was contained in nasal polyps from the patients of CRSwNP with AERD and it correlated with tissue eosinophilia [10]. Moreover, *PAF* contributes to mast cell activation, often linked to severe anaphylactic responses [9]. Its role in inducing vascular permeability leads to nasal hyperreactivity and congestion [11–13].

*PAF* is tightly regulated and produced by two distinct pathways 4 with 1) de novo pathway, which constitutively produce low levels of *PAF* and 2) remodeling pathway, which rapidly synthesize the majority of *PAF* removing fatty acids (typically arachidonic acid) from the sn-2 position of membrane phospholipid phosphatidylcholine (PC), which results in *lyso-PAF*, following to convert *lyso-PAF* into *PAF* by *LPCAT1* and *LPCAT2* [14,15]. The reverse reaction from *lyso-PAF* to PC is catalyzed by *LPCAT1–4* acyltransferase activity [16]. *PAF* is synthesized by a variety of cells including endothelial cells, platelets, macrophages, monocytes, neutrophils, and eosinophils [17], and shows its biological effects at high-picomolar concentrations, such as the typical serum concentration of *PAF* of 400 pg/ml in healthy individuals [18].

Produced *PAF* is rapidly degraded to *lyso-PAF* by *PAF* acetylhydrolases (*PAFAHs*), a family of Ca<sup>2+</sup>- independent lipoprotein-associated phospholipase A<sub>2</sub>. Half-life of *PAF* ranges from 3–13 min because *PAFAHs* regulate *PAF* activity by hydrolyzing *PAF* to *lyso-PAF* as a biologically inactive form [17]. *PAFAHs* have three isoforms and one isoform is a secreted form (Phospholipase A<sub>2</sub> Group VII(*PLA2G7*)) and two isoforms are intracellular forms (*PAFAH Ib* and *PAFAH II*) [19]. *PLA2G7* and *PAFAH II* can non-specifically hydrolyze oxidatively fragmented phospholipids with potent biological activities, while *PAF-AH Ib* displays high specificity for the sn-2 acetyl group of glycerophosphocholine (GPC) including *PAF* [20]. On the other hand, there was no report about the association between allergic conditions and *PLA2G7* activity, although increased *PLA2G7* activity was reported in various pathologic conditions, including ischemic stroke, myocardial infarction, familial HDL deficiency, chronic cholestasis, diabetes mellitus, rheumatoid arthritis, essential hypertension, and peripheral vascular disease [20]. Therefore, currently *PLA2G7* is thought to be separated from the *PAF*-metabolism associated genes in allergic conditions.

*PAF* effects are mediated by binding to the *PAF* receptor (*PTAFR*), leading to mobilization of intracellular calcium and activation of kinases. Activation of *PTAFR* leads to the activation of cytoplasmic *PLA2* with subsequent formation of leukotrienes, prostaglandins, and thromboxane after the cleavage of arachidonic acid [19]. *PTAFR* is expressed in T lymphocytes, monocytes and

macrophages, platelets, tracheal epithelium, vascular endothelium, lung alveolar wall, liver, small intestine, heart, skeletal and smooth muscle, brain microglia and neurons, myometrium, spleen, and kidney [21,22]. *PTAFR* expression is regulated by intracellular cyclic AMP, which can downregulate *PTAFR* gene expression and reduce *PAF*-induced arachidonic acid release [23,24].

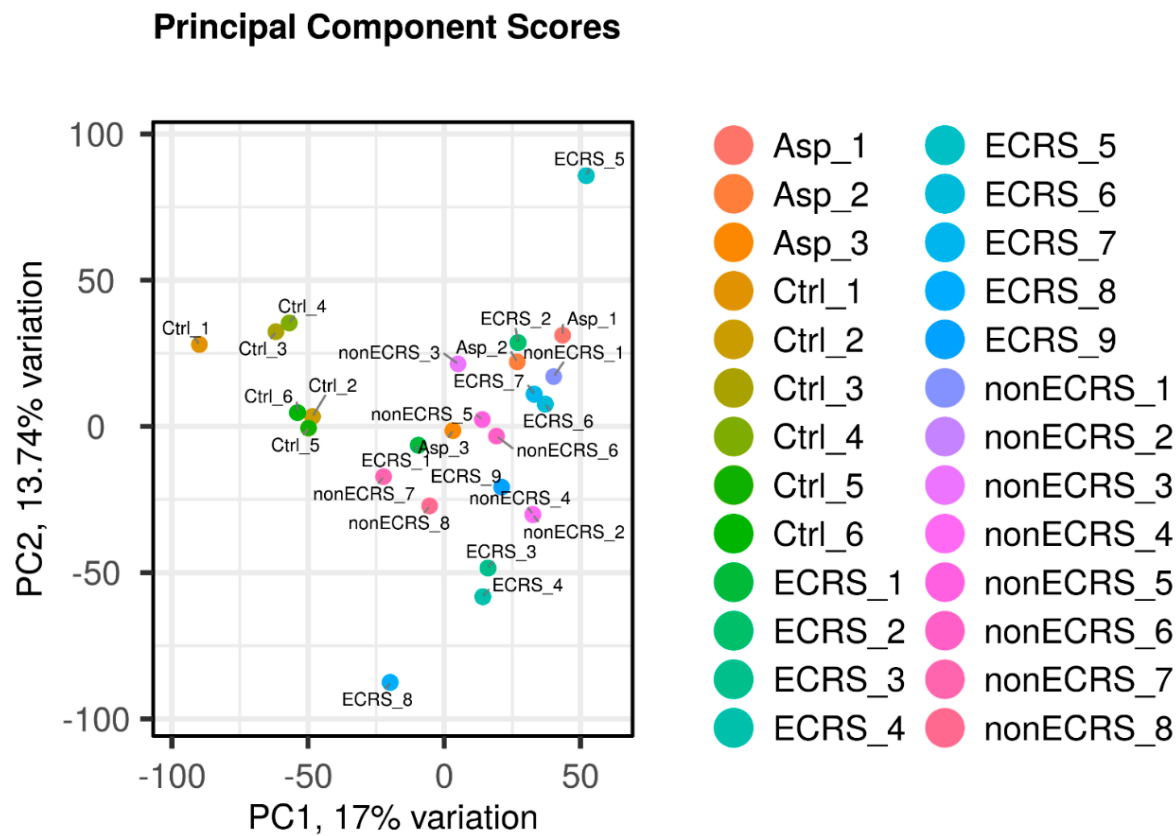
Stimulation of the *PTAFR* induces production of nitric oxide [25], histamine release from basophils, activation and degranulation of mast cells, chemotaxis of mast cells and eosinophils, recruitment of neutrophils, production of *IL-4* by B lymphocytes, bronchial smooth muscle contraction, and mucus secretion as well as increase vascular permeability [19]. The role of *PAF* in the pathogenesis of allergic rhinitis was currently demonstrated [26], but the role of *PAF* in the pathogenesis of CRSwNP was underestimated. The intricate relationship between *PAF* and the pathophysiology of CRSwNP, which include ECRS with AERD often showing anaphylactic reaction after administration of NSAIDs, suggests a potential link between *PAF* metabolism and its pathophysiology including the formation and maintenance of nasal polyps derived from vascular permeability associated with plasma extravasation [11–13,27].

Current classification methods like the JESREC criteria attempt to stratify ECRS based on clinical findings and eosinophil count [1], transcriptome analyses have revealed that the expression level of type 2 inflammatory cytokine and chemokine do not align with these clinical classifications [28]. These analyses have unveiled significant differences between the clinical classification and the severity of type 2 inflammation. Therefore, this study aims to explore and elucidate the complexities of *PAF* metabolism-associated gene expressions of key enzymes involved in *PAF* synthesis (*LPCAT1*, *LPCAT2*, *LPCAT3*, *LPCAT4*) and degradation (*PAFAH1B2*, *PAFAH1B3*, *PAFAH2*), along with *PAF* receptor (*PTAFR*), across subtypes classified as nonECRS and ECRS including ECRS with AERD(Asp) by clinical classification and hierarchical analysis-based classification. Understanding the significance of *PAF* in orchestrating eosinophil recruitment and its association with type 2 inflammation sets the stage for investigating how variations in *PAF* metabolism-associated gene expressions align with these subtypes. Identifying disparities in *PAF* metabolism-associated gene expressions could be crucial in comprehending the heterogeneity of CRSwNP and potentially guiding tailored therapeutic interventions. This investigation seeks to bridge the gap between transcriptomic variations, inflammatory phenotypes, and *PAF*-related molecular signatures, aiming to illuminate the intricate interplay between *PAF* metabolism and CRSwNP subtypes and/or cluster-based classification. Ultimately, this study aims to unravel the diverse landscape of *PAF*-associated gene expressions across distinct CRSwNP subtypes and/or cluster-based classification providing deeper insights into the molecular foundations contributing to the varying inflammatory profiles observed in this condition.

## 2. Results

### 2.1. Gene expression profiles of NPs between ECRS including Asp and nonECRS.

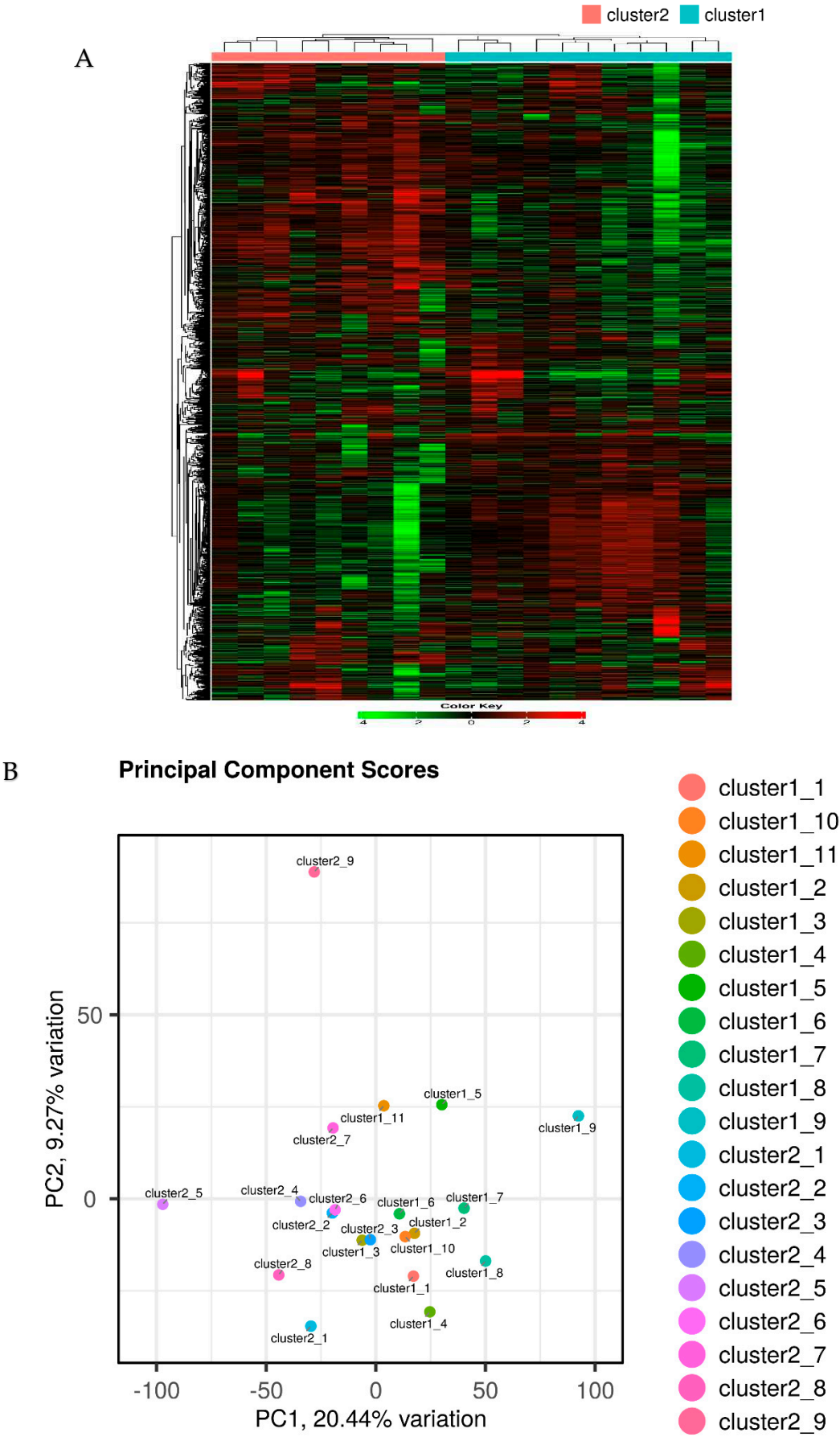
2.1.1 Principal Component Analysis (PCA), heatmap of the correlation matrix, and hierarchical cluster analysis PCA was conducted across all groups, indicating distinct segregation only for the control group, while ECRS, Asp and nonECRS did not show clear separation from each other (Figure 1). These findings support the rationale for subsequent analyses of *PAF* metabolisms, considering ECRS and Asp as part of the same ECRS group.



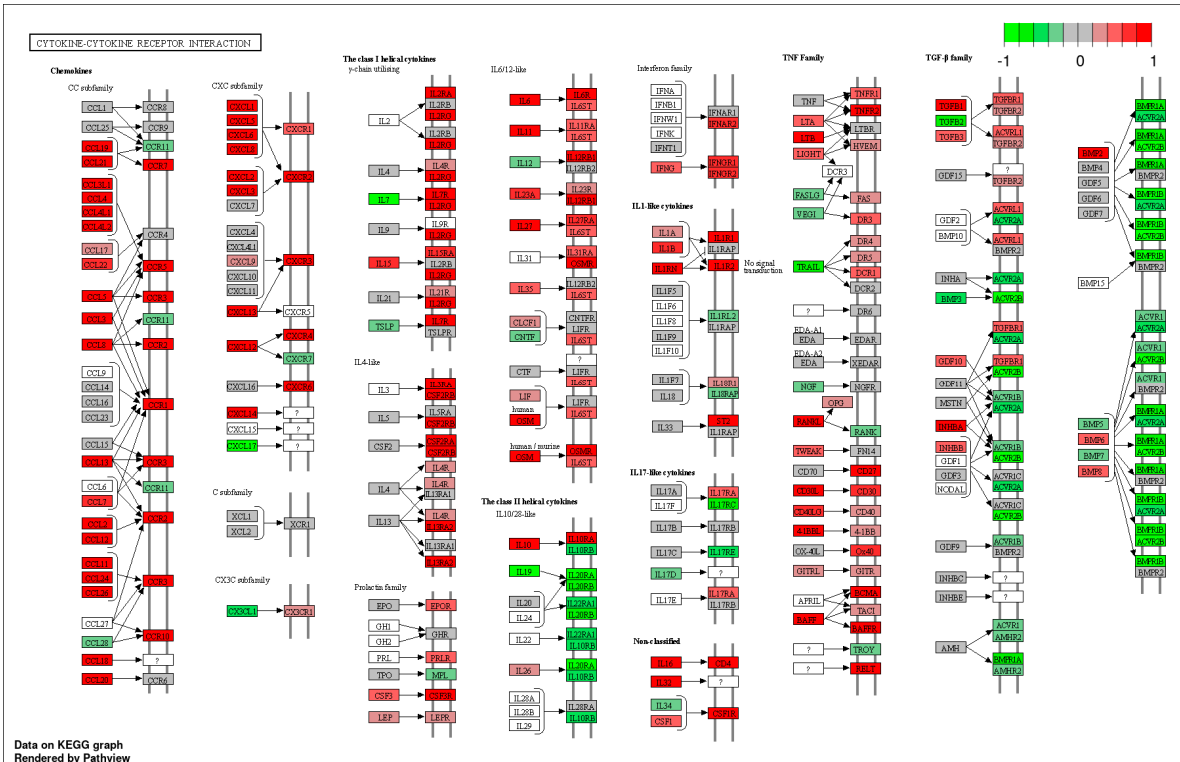
**Figure 1.** PCA performed among all groups. The control group was clearly segregated, but ECRS, nonECRS, and Asp were not segregated from each other.

2.2. Segregation of all CRSwNPs into two clusters using hierarchical cluster analysis

Hierarchical cluster analysis divided all CRSwNPs into two distinct clusters (referred to as cluster 1 and cluster 2) (Figure 2). However, no correlation was found between the clusters and the presence of asthma as a comorbidity. Notably, these two clusters exhibited significant variations in Differentially Expressed Genes (DEG) related to a wide array of cytokines and chemokines including interleukine (IL)-4 like cytokines, Chemokine (C-C motif) ligand (CCL26), and leukotriene B (LTB), indicating the differing severity of type 2 inflammation (Figure 3).



**Figure 2.** Hierarchic cluster analysis with all CRSwNPs segregated into two cluster (cluster1 and 2) among these CRSwNPs (A). Segregation of two clusters and comorbidity of asthma were not correlated. The new clusters and control showed clear separation in the PCA analysis (B).



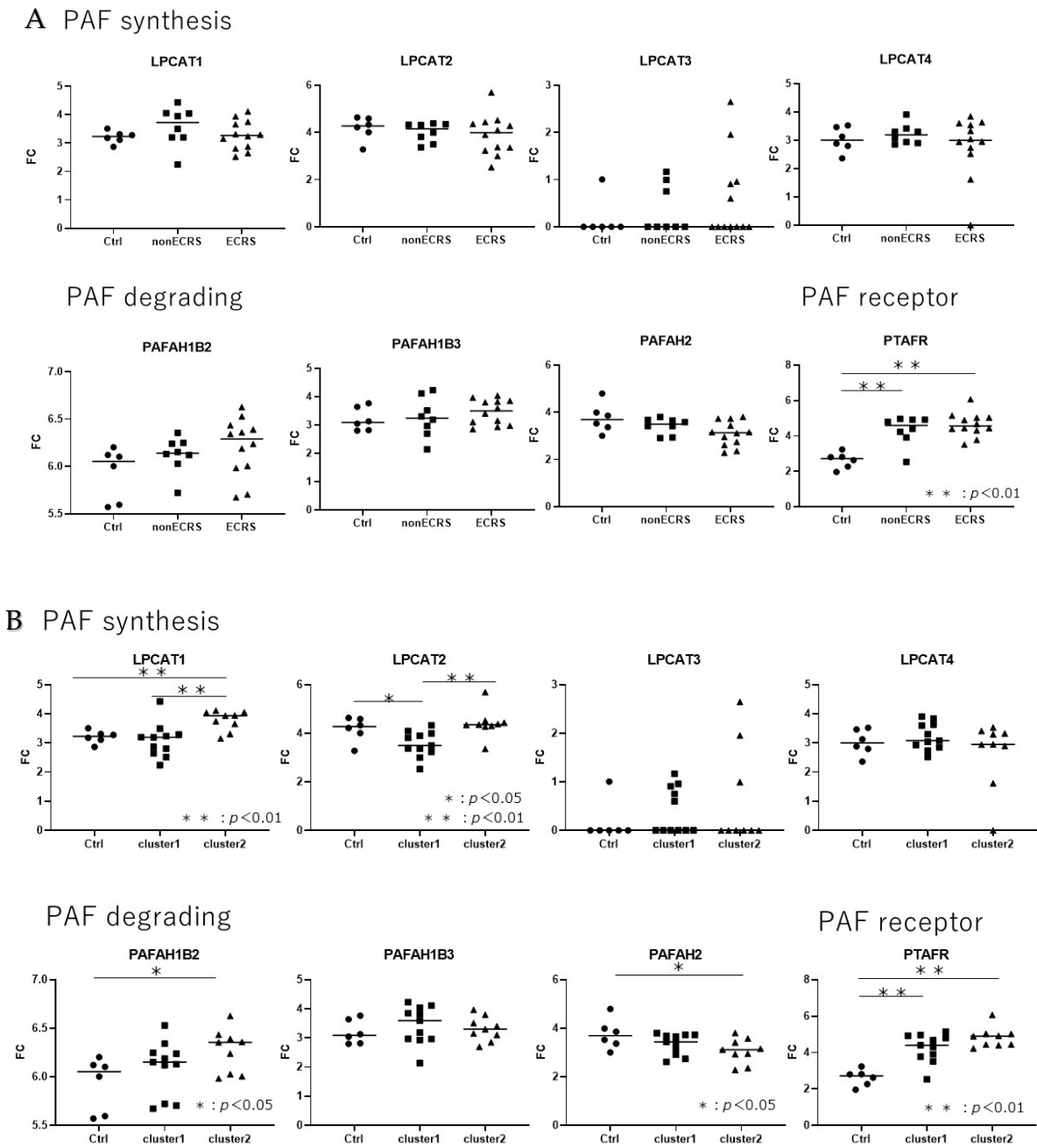
**Figure 3.** KEGG pathway analysis of cluster 1 vs cluster 2 in “cytokine-cytokine receptor interaction” by iDEP1.13. Red represent the upregulated DEGs and Green represent the downregulated DEGs in cluster 2 compared to cluster 1. Intense result of DEGs including upregulation of interleukine (IL)-4 like cytokine, Chemokine (C-C motif) ligand (CCL26), and leukotriene B (LTB) and suggested that cluster 2 is a severe group of type 2 inflammation.

2.3. Gene expression analysis about PAF-metabolism associated gene based on the JESREC-based clinical classification and clusters reflecting the severity of type 2 inflammation.

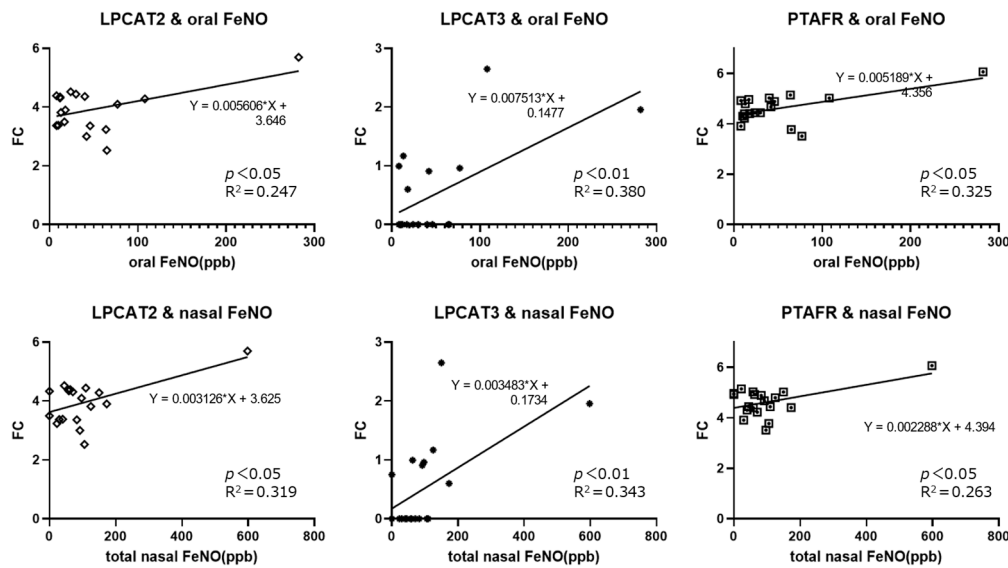
Each group of CRSwNP as nonECRS and ECRS including Asp and Ctrl showed no significant difference in LPCAT1, LPCAT2, LPCAT3, LPCAT4, PAFAH1B2,PAFAH1B3, and PAFAH2, but both groups of CRSwNP were upregulated in PTAFR compared to Ctrl (Figure 4). Each clusters reflecting the severity of type 2 inflammation showed no significant difference in LPCAT3, LPCAT4, and PAFAH1B3. Although cluster1 showed upregulated PTAFR and downregulated LPCAT2 compared to Ctrl, cluster2 showed upregulated LPCAT1, PAFAH1B2, and PTAFR, and downregulated PAFAH2. Compared between each clusters, cluster2 was upregulated in LPCAT1 and LPCAT2.

2.4. Gene expression analysis between PAF-metabolism associated gene and clinical features.

Significant correlation only between oral FeNO or total nasal FeNO and LPCAT2, LPCAT3, and PTAFR were recognized in CRSwNP (Figure 5), but there were no significant correlation between both FeNO and LPCAT1, LPCAT4, PAFAH1B2, and PAFAH1B3 (data not shown). There were also no correlation between tissue eosinophil, blood eosinophil, total IgE, and JESREC score and each genes expressions of LPCAT1, LPCAT2, LPCAT3, LPCAT4, PAFAH1B2,PAFAH1B3, PAFAH2, and PTAFR (data not shown).



**Figure 4.** PAF metabolism-associated gene expression(FC: fold change) in clinical classification with JESREC criteria (A) and clusters enhancing the severity of type 2 inflammation (B). Clinical classification did not show a clear difference between the phenotype of CRSwNP but the clusters enhancing endotype of CRSwNP showed clear difference in PAF metabolism-aassociated gene expression.



**Figure 5.** PAF metabolism-associated gene expression (FC: fold change) in CRSwNP based on the test of oral FeNO and total nasal FeNO. Only oral FeNO and total nasal FeNO collerated with LPCAT2, LPCAT3 and PTAFR, but all of the data showed low R<sup>2</sup> in linear regression analysis.

### 3. Discussion

PAF-metabolism associated gene expression using transcriptome analysis revealed that clinical classification based on the JESREC study showed the significant upregulation of *PTAFR* gene expression in nonECRS and ECRS compared to Ctrl, but the other genes did not show any difference among nonECRS, ECRS and Ctrl. Additionally, there was no difference between nonECRS and ECRS in *PTAFR* gene expression. These results showing significant upregulation in nonECRS and ECRS compared to Ctrl and no difference between nonECRS and ECRS in *PTAFR* gene expression were also previously demonstrated [27] and these results suggested that clinical classification based on the JESREC criteria or the status with/without comorbid asthma and/or AERD may not detect the difference of PAF-metabolism in each clinical classification because PCA analysis with nonECRS, ECRS, and Asp could not segregate each other. On the other hand, upregulation of *PTAFR* gene expression in nasal polyp from both nonECRS and ECRS suggests that *PTAFR* upregulation enhancing the possibility of PAF-signaling upregulation associate with the formation or maintenance of nasal polyp.

However, two clusters from hierarchical analysis could clearly segregate from each other in PCA analysis and showed the clear difference in variety of cytokines expression in KEGG pathway analysis suggesting the reflection of the severity of type 2 inflammation. In the cluster analysis of PAF-associated gene expression, cluster 2 showed significant upregulation of *LPCAT1*, *PAFAHB2*, and *PTAFR*, and significant downregulation of *PAFAH2* compared to Ctrl. In contrast, cluster 1 showed significant upregulation of *PTAFR* and significant downregulation of *LPCAT2*. Compared between cluster 1 and 2, cluster 2 was significantly upregulated in *LPCAT1* and *LPCAT2* expression.

*LPCAT1* and *LPCAT2* are main enzymes of PAF synthesis from *lyso*-PAF [14,15], and *PAFAH2* is main degrading enzymes of PAF by hydrolyzing PAF to *lyso*-PAF as a biologically inactive form [19]. Although *PAFAH1B2* and *PAFAH1B3* are capable of hydrolyzing PAF, previous report demonstrated that RNAi-mediated knockdown of *PAFAH1B2* or *PAFAH1B3* does not alter PAF levels or PAF hydrolytic activity, indicating that these enzymes may possess alternate endogenous substrates [29]. These results suggest that cluster2, which can reflect the severity of type 2 inflammation, have high PAF-associated pathophysiology with upregulation of PAF synthesis and downregulation of PAF

degrading leading to the local *PAF* accumulation and intensify the effects of *PAF* signaling via upregulation of *PTAFR*.

The result showed for the first time that severe type 2 inflammation induce the high *PAF*-associated pathophysiology and the main cause of the high *PAF*-associated pathophysiology assumingly comes from high *lyso-PAF* and *PAF* synthesis via overexpressed *LPCAT1* and *LPCAT2* compared to the cluster of moderate to low type 2 inflammatory type of CRSwNP (cluster 1). On the other hand, our result did not show any correlation between each gene expressions associating *PAF* metabolism and blood/tissue eosinophil count or the clinical classification. Only the oral FeNO and total nasal FeNO showed the relationship between *LPCAT2*, *LPCAT3*, and *PTAFR*, but could not exhibit the above differences of *PAF*-metabolism associated gene expression, especially in *LPCAT1* and *LPCAT2*, shown in cluster analysis. The difference may come from the discrepancy between the severity of type 2 inflammation and blood/tissue eosinophilia, both FeNO level, or clinical classification because blood/tissue eosinophil count is used for the biomarker of eosinophilia [30] and FeNO is for airway inflammation or irritation [31] and clinical classification is for the phenotype diagnosis [1]. Although oral FeNO and total nasal FeNO reflect the severity of allergic inflammation [31], our result revealed that both FeNO can reflect the severity of allergic inflammation but not the severity of type 2 inflammation. Direct biomarker for the severity of type 2 inflammation, which have been underdeveloped yet, should be made for the further analysis of pathophysiology in CRSwNP.

Previous report mentioned that difference of *lyso-PAF* concentration between normal nasal polyp and nasal polyp having AERD suggested the association between airway inflammation and/or disease severity and *lyso-PAF* concentration [27]. Other paper also mentioned that human nasal polyp with severe eosinophil infiltration showed high *PAF* concentration [10]. Our result also clearly showed that the group of nasal polyps with severe type 2 inflammation had the increased *PAF*-metabolism, which can play an important role in the pathogenesis of CRSwNP inducing increased tissue neutrophilia and eosinophilia in nasal mucosa as a function of *PAF* signaling [19]. Therefore, these results may enhance that *PAF*-metabolism associated gene expression, especially in combination of *LPCAT1* and *LPCAT2* gene expression, can become a biomarker for the severity of type 2 inflammation in CRSwNP.

In summary, this study demonstrates the upregulation of *PTAFR* gene expression in nasal polyp and severe type 2 inflammation leads to increase high *PAF*-metabolism in nasal polyp, which may induce the proliferation and maintenance of nasal polyp. However, due to the classification using hierarchical analysis, small sample size, and only from Japanese patients, further research is needed to confirm our findings and the association between type 2 inflammation and the production of *PAF*-metabolism associated protein including *PAF* and/or *lyso-PAF* to reach the development of anti-*PAF* therapies for CRSwNP especially with severe type 2 inflammation. Additionally, the research about *LPCAT1* and *LPCAT2* gene expression in CRSwNP should be performed to confirm whether these gene expression become a good biomarker for the severity of type 2 inflammation or not.

## 4. Materials and Methods

### 4.1. Patient recruitment

Patients with or without CRS, who underwent endoscopic sinus surgery in Hiroshima Medical University Hospital between October 2016 and October 2021, were enrolled. The diagnosis of CRS was based on computed tomography (CT) scanning, patient history, clinical symptoms, and endoscopic findings. The inclusion criteria for CRSwNP were as follows: treatment without oral/nasal steroids within 4 weeks before surgery, no improvement in continuous nasal drip, post-nasal drip and nasal congestion after medical treatment including low-dose macrolide therapy. Patients with CRSwNP were clinically diagnosed as ECRS or nonECRS based on the diagnostic criteria of the JESREC study [4]. The diagnostic criteria for ECRS (JESREC score) include 1) side affected: both sides; 3 points, 2) with nasal polyps; 2 points, 3) CT changes: ethmoid/maxillary  $\geq 1$ ; 2 points, and 4) peripheral blood eosinophil count (%):  $2 < \text{and} \leq 5\%$ ; 4 points,  $5 < \text{and} \leq 10\%$ ; 8 points,  $10\% <$ ; 10 points. Eosinophil infiltration into the NPs was diagnosed by calculating the mean cell count of the 3 most

dense areas of eosinophils in the hematoxylin and eosin (HE) stained sections under x400 magnification (ocular lens, field number 22). A diagnosis of ECRS was made by both the total JESREC score of 11 points or higher and eosinophil infiltration count into the NPs of 70 or more [4]. The group of ECRS with aspirin exacerbated respiratory disease was independently classified as Asp (N=3), and the others were classified as ECRS (N=9) and nonECRS (N=8). Control patients (Ctrl) (N=6) were also diagnosed based on the anatomical abnormalities but showed no inflammatory mucosal change or bacterial infection around the uncinate process. The exclusion criteria for CRSwNP were as follows: treatment with oral/nasal steroids within 4 weeks before surgery, fungal/allergic fungal rhinosinusitis, and primary ciliary dyskinesia. Demographics and clinical characteristics were obtained from the medical records. The detailed patient demography is shown in Table 1.

**Table 1.** Patient demographics. BMI: body mass index, NP: nasal polyp.

patients	control	control	control	control	control	control	nonECRS_1	nonECRS_2	nonECRS_3	nonECRS_4	nonECRS_5	nonECRS_6	nonECRS_7	nonECRS_8	ECRS_1	ECRS_2	ECRS_3	ECRS_4	ECRS_5	ECRS_6	ECRS_7	ECRS_8	ECRS_9	Asp_1	Asp_2	Asp_3
cluster							2	1	2	1	1	2	1	1	1	2	1	1	2	2	2	1	1	2	2	1
height	153.4	149	158	151.6	145	151.7	165	175.2	162.4	156.8	172	159.5	166.6	169	173.7	154.2	166.9	146.7	172.2	168	145.5	162.1	162	162	156.5	150
weight	52.3	46.6	58.8	53	58.8	41.6	64.2	77.8	49.4	54.4	69.5	61.7	67.9	61	80.5	55.9	57.3	40.1	57.9	62.7	68.3	44	64.5	50	46.3	49.7
BMI	22.2	21.0	23.6	23.1	28.0	18.1	23.6	25.3	18.7	22.1	23.5	24.3	24.5	21.4	26.7	23.5	20.6	18.6	19.5	22.2	32.3	16.8	24.6	19.1	18.9	22.1
sex	F	F	F	F	F	F	M	M	F	F	M	M	M	F	M	F	M	M	F	M	F	F	M	F	F	F
age	64	66	39	70	61	69	53	44	66	67	47	54	69	40	43	52	51	63	31	48	61	47	70	60	42	67
asthma (1:positive)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1
olfactory disturbance (1:positive)	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1
smoke (1:positive)	0	0	0	0	0	0	0	ex	0	0	0	1	0	0	ex	0	1	1	0	0	0	0	0	0	0	0
blood eosinophil (%)	0.9	1.5	0.6	0.2	1.6	1.2	1.3	2.9	0.3	0.3	1.6	1.6	3.6	1.6	11.6	10.2	5.5	1.9	9.6	19.5	1.1	6.1	0.6	20	11.2	10.1
total IgE	467	85.6	NA	142	NA	NA	477	73.2	22	54.8	14.9	860	506	15.6	319	208	444	536	24.3	66.7	122	72.6	255	71.1	462	212
total NP score							0	5	2	8	3	0	2	5	4	2	6	6	6	6	6	6	2	7	6	6
total CT score							11	10	4	24	12	6	16	17	16	10	10	16	9	24	22	24	21	19	27	26
tissue eosinophil of nasal polyp (cell count/HPF)							12.0	6.6	15.0	38.0	70.3	0.0	NA	6.0	76.0	73.0	110.0	83.0	58.3	220.0	117.0	145.0	140.0	416.0	170.0	170.0
JESREC score							3	9	0	5	7	0	9	5	17	17	15	11	15	15	11	15	17	17	17	17
oral FeNO(ppb)							8	13	12	17	NA	12	27	8	77	24	65	10	282	46	40	42	18	30	108	64
total nasal FeNO(ppb)							63	125	71	0	0	59	57	30	97	45	106	40	598	83	58	92	173	110	150	23

4.2. RNA-seq using BRB-seq

BRB-seq [32] was performed for library preparation with the following modifications. Barcoded oligo-dT based primer (5'-GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNNNNNNCCCCCCCCCTTTTTTTTTTTTTTTTTTTTTTTT-3' ; 10bp "N"=UMI, 9bp "C"=cell barcode) was used for single-stranded synthesis and Second Strand Synthesis Module (NEB, #E6111) was used for double-stranded cDNA synthesis. In-house MEDS-B Tn5 transposase [27,28] was used for tagmentation and libraries were amplified for 10 cycles of PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, #M0530) with the following primers (5'-AATGATACGGCGACCGAGATCTACACindexGTTTCAGAGTTCTACAGTCCGA-3',5'-CAAGCAGAAGACGGCATACGAGATindexGTCTCGTGGGCTCGGAGATGT-3'). An Illumina NovaSeq6000 was used to obtain 15bp of barcode read (Read1) and 81bp of insert read (Read2).

4.3. Data processing of BRB-seq

Read1(barcode read) was extracted by using UMI-tools(ver.1.1.1) with the command "umi\_tools extract -I read1.fastq --read2-in=read2.fastq --bc-pattern=NNNNNNNNNNNCCCCCCCCCCC --read2-stdout ". Adaptor sequences and low-quality sequence were removed and read length below 20 bp were discarded using Trim Galore (ver.0.6.7). Reads were mapped to the GRCh38 reference using HISAT2 (ver.2.2.1). Read counts for each gene were obtained by featureCounts (ver.2.0.1), and UMI duplication was removed by UMI-tools with the command "umi\_tools count --method=unique --per-gene --per-cell --gene-tag=XT". Normalized read count value was obtained by using DESeq2 (ver.1.34.0). Gene-level expression data (read counts) were processed by using the web portal for integrated differential expression and pathway analysis (iDEP.1.13; <http://bioinformatics.sdstate.edu/idep11>). iDEP.1.13 was used for principal component analysis (PCA), hierarchical clustering with a heatmap by center genes, identification of differentially expressed genes (DEGs), and pathway analysis. DEGs were extracted with an FDR cutoff of 0.1 and min-fold change of 2.

Pathway analysis was performed using GAGE with hsa04060 cytokine-cytokine receptor interaction of Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Geneset size was set at minimum 5 and maximum 2000, and pathway significance cutoff (FDR) was set at 0.2.

#### 4.4. Data analysis for PAF metabolism associated gene expression

In the category of fold change in CRSwNP, PAF synthesis (LPCAT1, LPCAT2, LPCAT3, LPCAT4) and degradation (PAFAH1B2, PAFAH1B3, PAFAH2), along with the PAF receptor (PTAFR) were measured and classified with clinical classification and hierarchical classification. Statistical analysis was performed using Welch's t test and Pearson correlation coefficient with Graphpad prism 8.4.3.

## 5. Conclusions

This study revealed and shed light on the PAF metabolism in endotype categorization of the severity of type 2 inflammation. Additionally, this study also revealed that hierarchical analysis-based classification as endotype categorization was useful to elucidate the differences of CRSwNP. This result may provide a valuable data for further studies to elucidate the differences of endotype of CRSwNPs, future therapy for CRSwNP using anti-PAF metabolism drug, and possible biomarker of *LPCAT1* and *LPCAT2* for severity of type 2 inflammation.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Hiroshima University Hospital, Hiroshima University, Hiroshima, Japan (Hi-136; August.11.2014 as date of approval).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

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