SUPPLEMENTARY MATERIAL FOR ONLINE REPOSITORY

TEXT:

METHODS

Study Design and Population

This study was approved by the Vanderbilt University Institutional Review Board. Patients presented to the Vanderbilt Asthma, Sinus, and Allergy Program (ASAP) and Otolaryngology clinic at the Vanderbilt Bill Wilkerson Center. CRS was diagnosed according to the European Position Paper on Rhinosinusitis and Nasal Polyps and the International Consensus Statement on Allergy and Rhinology and therefore were initially managed medically [1, 2]. Patients with continued symptoms who elected to undergo endoscopic sinus surgery were prospectively enrolled. Only patients with diffuse, bilateral inflammatory CRS were included, and patients with odontogenic rhinosinusitis, fungus balls, and isolated osteomeatal complex obstruction were excluded. Control cases included patients undergoing pituitary or skull base surgery without a clinical or radiographic history of CRS. Patients were excluded if they had received systemic steroids within 4 weeks of surgery. Patients with cystic fibrosis, autoimmune, or granulomatous diseases or who were receiving immune-directed monoclonal antibodies were excluded. The presence of concomitant allergic rhinitis and asthma was recorded. Allergic rhinitis was diagnosed based on positive skin prick testing and/or prior physician diagnosis and clinical history suggestive of seasonal variation of atopic symptoms with improvement following use of topical nasal steroid or oral antihistamines. Asthma was diagnosed based on a positive methacholine challenge or consistent pulmonary function studies, or by prior diagnosis by a pulmonologist. Patient reported symptom severity was measured utilizing the Sinonasal Outcome Test-22 (SNOT-22) [3]. All patients underwent a high resolution CT scan of the paranasal sinuses within 3 months of surgery. Each scan was evaluated by two physicians who were blinded to subject identifiers and diagnosis. A standard Lund Mackay scoring system was used to assess overall extent of CRS. Subjects enrolled in the study also completed the 40-

item Smell Identification Test (SIT) immediately prior to surgery. The SIT has excellent sensitivity, correlates closely with scores attained via formal threshold testing, and has the advantage of being easily and quickly administered to subjects on the day of surgical intervention [4]. Raw scores were adjusted for patient age and gender by subtracting the mean normative age- and sex-appropriate SIT score from the total SIT score for each subject [5]. Thus a negative adjusted SIT score represents reduced sense of smell compared to the mean for that subject's age and gender. Normative SIT scores were extracted from the Smell Identification Test Administration Manual (Sensonics International; Haddon Heights, NJ).

Mucus Collection and Histopathologic Evaluation of Sinonasal Tissue

At the beginning of surgery, 9 x 24mm polyurethane sponges (Summit Medical; St. Paul, MN) were placed bilaterally into the middle meatus or ethmoid cavity of each subject under endoscopic guidance as previously reported [6]. This approach has been previously validated, and has advantages over other methods for mucus collection, including standardization between subjects and avoidance of specimen dilution [7-9]. Each sponge was removed after 5 minutes, 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 x g for 10 minutes to elute mucus. Samples were then combined, gently vortexed, and again centrifuged for 5 minutes to remove any cellular debris. Supernatants were removed, placed into a new microcentrifuge tube, and frozen at -80°C for later analysis.

Cytokine assays were performed using a multiplex cytokine bead assay (BD Biosciences; Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, 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. After addition of 1 mL wash buffer, samples were centrifuged at 200 x g for 5 minutes 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). Data was analyzed using BD FCAP Array Software version 3.0.

Sinonasal tissue was collected from the ethmoid bulla or posterior ethmoid sinus in all patients undergoing endoscopic sinus surgery for CRS. Tissue from healthy controls was collected from either the ethmoid sinus or sphenoethmoid recess. Histopathological evaluation of excised tissue was performed by a pathologist in a blinded fashion and the mean number of eosinophils counted over 5 randomly selected high powered fields (HPF) was recorded.

Statistics

Sample size for principal component analysis and subsequent clustering was estimated by establishing a subject to variable ratio of 5:1 (90 subjects, 18 biological variables) as recommended by Gorsuch and Hatcher [10, 11]. Adequacy of the sample size was verified *post hoc* by assessing variable communality (heavy loading of variables in retained components). 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 natural logarithm, resulting in elimination or significant reduction of skewing for all variables. A principal component factor analysis with varimax rotation was then performed on the transformed biological variables. Variables with a loading > 0.5 were retained. The appropriate number of factors was selected by analysis of the Scree plot, with a requirement that retained factors explain at least 70% of data variance, and that each factor have an eigenvalue > 1.0. The regression method was then used to calculate a factor score for each subject in each of the five factors. Hierarchical cluster analysis was performed using Ward's method on squared Euclidian distances using the five factor scores. The hierarchical structure of the data was visualized using a dendogram. The appropriate number of clusters was selected by calculating within and between class variance for models that included between 2 and 15 clusters, with a goal of minimizing within class variance and maximizing between class variance. This was also verified by identifying the bend on the accompanying Scree plot.

Clusters were then retrospectively compared against the individual components used for analysis, and then against the individual biological variables themselves. Subsequently, clusters were compared against demographic and clinical data. For comparison between groups, normality of data was assessed using the D'Agostino-Pearson omnibus test. Variables with a normal distribution were compared using a student's t-test or analysis of variance, while nonparametric data was analyzed using the Mann-Whitney test or Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Comparative data was presented as means +/- standard deviation or medians with interquartile range, respectively. A p value of 0.05 was considered statistically significant for all comparisons. Statistical analyses were performed with Prism 6 software (Graphpad; La Jolla, CA), and principal component and hierarchical cluster analysis were performed using SAS (SAS Institute Inc.; Cary, NC) and XLSTAT (Addinsoft; New York, NY.).

TABLES:

Table E1. **Study population and demographics for healthy control and CRS patients**. Data is presented as frequencies (percentages), means +/- standard deviation or medians with interquartile range. AERD, aspirin-exacerbated respiratory disease; AFRS, allergic fungal rhinosinusitis; BMI, body mass index; CT, computed tomography; NCS, nasal corticosteroid; LTR, anti-leukotriene; SIT, smell identification test; SNOT-22, sinonasal outcome test-22.

Table E2**. Loading of biological variables after principal component analysis.** Values for all biological variables were transformed to achieve normalcy and then analyzed with principal component analysis with varimax rotation. Variables with a loading > 0.5 were retained. Data for the first five factors are shown based on analysis of the plotted Eigenvalues for each component. The 5-factor solution explained 71.3% of the collective data variance.

Table E3**. Differences in mucus cytokines levels between the 6 identified CRS endotypes.** Median cytokine levels of control subjects and each of the 6 CRS clusters are shown for all 18 assayed biological variables. Significant differences among CRS endotypes and between each CRS endotype and the control group were identified using the Kruskal-Wallis test. Differences among the endotypes are represented by the listed p-values, with a p-value < 0.05 considered statistically significant. Post hoc analysis of differences between each cluster and healthy controls were then performed. Clusters with a higher (*) or lower (¥) median cytokine level compared to controls are annotated. Data is represented as medians with interquartile range. **BOLD**, $p < 0.05$.

FIGURES:

Figure E1. **Validation of the number of principal components and clusters.** (A) Eigenvalues for calculated factors in a Scree plot. The optimum number of factors was estimated by identifying an approximate break point on the plotted curve and by eliminating factors with an eigenvalue of less than 1. (B) The ideal number of CRS clusters was determined by comparing within and between class variance for models that included between 2 and 15 clusters, with a goal of minimizing within class and maximizing between class variance.

Figure E2. **Postoperative improvement in sinonasal quality of life among high- and low-inflammation clusters.** (A) Clusters were combined by defining a higher 'cut point' on the dendogram. This corresponded to high- and low-inflammation clusters with distinct pathophysiology, as defined in Figure 3. Subjects in Clusters 3-6 were more likely to have had prior endoscopic sinus surgery than subjects in Clusters 1 and 2 (75.8% vs. 37.8%; $p < 0.001$) (B) and had a greater number of prior procedures (median 1.0 vs. 0.0, $p < 0.0001$) (C). Postoperative sinonasal quality of life, as assessed by the SNOT-22 questionnaire, was plotted for all subjects with postoperative follow-up of at least 6 months (D,E). Comparison of total (F) and % improvement (G) in SNOT-22 scores were than compared between each group. No significant difference in overall SNOT-22 improvement was identified between groups ($p = 0.12$), however Clusters 3-6 had a greater % improvement, compared to Clusters 1 and 2 ($p = 0.04$). Data for SNOT-22 and frequency of prior sinus surgery are presented as box-whisker plots with the median and range for each group.

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Table E1.

	Healthy Control	CRS
No.	17	90
Age (years)	50.5 +/- 13.1	48.5 +/- 13.1
Sex, no. (% female)	13 (76)	42 (47)
Race, no. (% white)	13 (76)	80 (89)
Current smoker, no. (%)	1 (6)	6 (7)
BMI (kg/m^2)	30.5 +/- 7.0	29.7 +/- 6.2
Nasal polyps, no. (%)	0 (0)	53 (59)
Asthma, no. (%)	0 (0)	42 (47)
Allergic Rhinitis, no. (%)	1 (6)	56 (62)
AERD, no. (%)	0 (0)	12 (13)
AFRS, no. (%)	0 (0)	10 (11)
Taking NCS, no. (%)	1 (6)	71 (79)
Taking LTR, no. (%)	0 (0)	25 (28)
SNOT-22 score	-	47.1 +/- 18.7
CT score	1.0 (0.0-3.6)	16.0 (11.0-20.0)
SIT score	-4.0(-7.0--1.0)	-7.0(-24.5--3.0)
Prior surgery, no. (%)	0 (0)	43 (48)

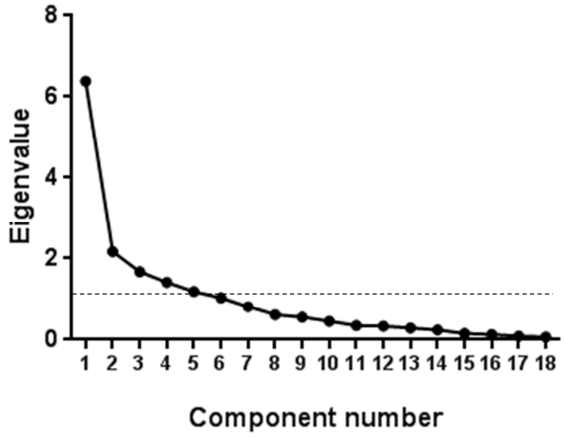
Table E2.

	Rotated Factor				
Variable	**1**	**2**	**3**	**4**	**5**
IL-1β	0.883				
IL-8	0.833				
IL-6	0.718				
TNF-α	0.631				
Eotaxin	0.604				
IL-7					
IL-5		0.943			
IL-13		0.912			
IL-9		0.655			
IL-2			0.76		
IL-21			0.681		
IL-4			0.553		
IL-12			0.551		
IFN-γ				0.926	
IL-10				0.894	
IL-3					0.823
IL-17					0.505
RANTES					0.504

Table E3.

	Control	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	p-value
No.	17	31	24	6	7	13	7	
IL-1β	42.1(13.3-211.7)	101.9(27.6-289.8)	7.1(1.5-12.5)¥	323.8(80.2-1491.0)	24.9(15.8-139.1)	549.1(171.6-6211.0)*	521.6(287.3-2845.0)	**<0.0001**
IL-2	0.0(0.0-11.1)	2.9(0.0-12.8)	0.0(0.0-1.6)	19.9(4.0-36.1)	17.8(11.3-46.9)*	0.0(0.0-7.1)	8.8(2.1-68.1)	**0.0001**
IL-3	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.4(0.0-1.2)	1.7(1.4-13.0)*	0.0(0.0-0.0)	0.0(0.0-0.1)	**<0.0001**
IL-4	0.08(0.0-1.30)	0.0(0.0-1.4)	0.0(0.0-0.0)	2.5(0.9-3.1)	4.2(1.4-4.4)*	0.1(0.0-2.3)	4.0(1.9-5.2)*	**<0.0001**
IL-5	0.12(0.01-0.77)	3.2(0.1-27.4)*	9.3(1.6-60.8)*	507.1(325.4-1843.0)*	160.6(40.0-240.5)*	1.2(0.1-10.4)	9.0(2.5-18.6)*	**<0.0001**
IL-6	59.8(13.4-190.3)	116.3(41.0-285.9)	9.8(4.4-48.0)	1701.0(1116.0-5092.0)*	205.8(42.6-659.7)	119.9(47.6-1138.0)	733.7(265.5-1076.0)*	**<0.0001**
IL-7	3.7(1.6-9.8)	5.4(1.6-11.3)	1.7(0.4-3.0)	14.8(12.7-18.9)	9.2(2.9-19.9)	7.4(2.2-20.5)	23.7(12.6-47.6)*	**<0.0001**
IL-8	3067(1610-9078)	5461(2838-9885)	1337(861-2428)	7838(3546-132655)	3264(1543-15683)	12840(4757-38650)	13001(10322-335288)*	**<0.0001**
IL-9	0.0(0.0-1.6)	0.0(0.0-0.0)	0.0(0.0-0.0)	23.2(8.6-64.6)*	16.6(0.9-63.4)*	0.1(0.0-3.1)	4.0(1.1-16.9)	**<0.0001**
IL-10	2.6(0.1-6.3)	4.0(0.9-8.2)	0.8(0.0-3.1)	27.1(15.0-63.2)*	10.6(6.9-17.5)	1.2(0.0-15.6)	9.9(6.6-23.3)	**<0.0001**
IL-12	43.5(20.3-117.7)	72.3(12.4-113.5)	3.4(0.0-4.9)¥	112.7(10.4-325.7)	52.2(20.2-87.2)	45.2(2.8-121.2)	355.6(188.7-681.0)*	**<0.0001**
IL-13	1.6(0.0-6.7)	3.0(0.0-22.0)	7.7(0.0-37.5)	468.6(346.4-712.9)*	86.6(36.8-358.8)*	2.9(0.6-7.8)	52.4(24.6-93.5)*	**<0.0001**
IL-17A	0.0(0.0-0.0)	0.0(0.0-0.8)	0.0(0.0-0.2)	4.0(3.2-8.3)*	9.2(4.9-14.5)*	2.1(0.0-8.1)	4.2(1.8-17.9)*	**<0.0001**
IL-21	59.6(0.0-155.3)	19.5(0.0-118.9)	7.0(0.0-12.0)	71.6(0.0-305.2)	69.0(9.9-88.2)	0.0(0.0-4.8)	243.9(102.0-653.9)*	**0.0001**
TNF-α	5.1(1.9-7.1)	5.0(2.8-11.6)	0.2(0.0-2.0)	59.7(7.4-156.0)	8.3(6.2-19.0)	9.7(0.1-12.9)	15.6(10.2-174.5)*	**<0.0001**
IFN-γ	0.0(0.0-1.4)	0.0(0.0-2.7)	0.0(0.0-0.8)	4.1(2.6-10.6)*	2.1(1.0-3.1)	0.0(0.0-1.1)	2.1(0.6-7.6)	**0.001**
Eotaxin	17.8(12.4-48.2)	14.3(5.6-32.1)	7.4(2.8-17.7)	112.7(76.5-303.5)*	9.7(7.6-11.0)	25.1(5.8-54.8)	49.1(28.3-107.6)	**0.0001**
RANTES	1848(654-2686)	4355(756-6423)	3107(764-8058)	2128(937-2590)	331(142-6902)	120(22-895)	3207(1171-6118)	**0.002**

A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6
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
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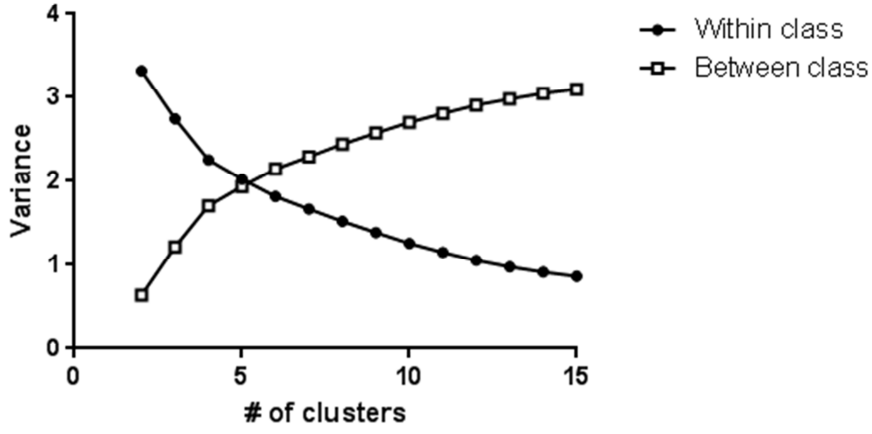
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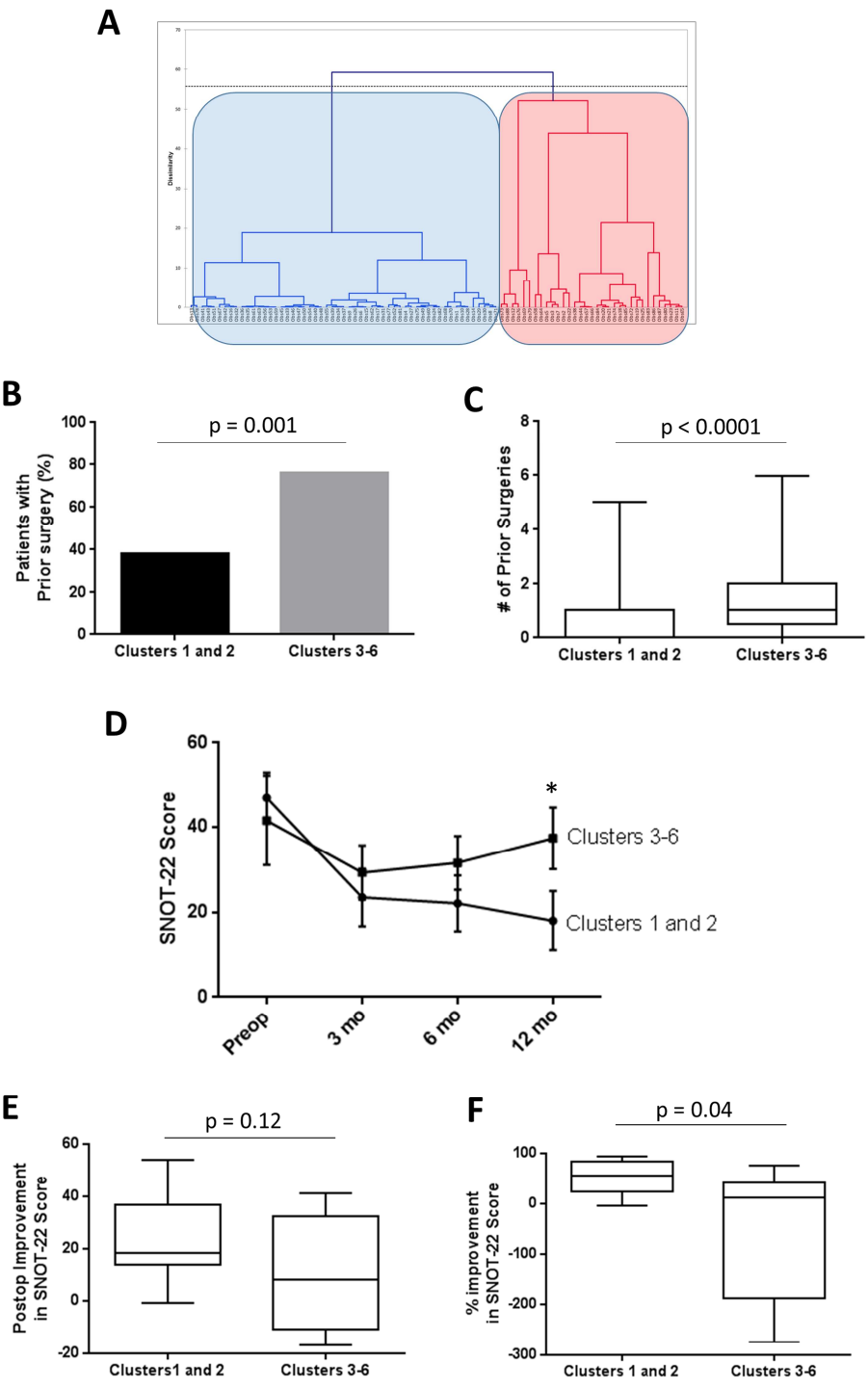
3
2
1
0
0
5
10
15
# of clusters
Within class
Between class


A
B
p = 0.001
Patients with Prior surgery (%)
100
80
60
40
20
0
Clusters 1 and 2
Clusters 3-6
C
p < 0.0001
# of Prior Surgeries
8
6
4
2
0
Clusters 1 and 2
Clusters 3-6
D
SNOT-22 Score
60
40
20
0
*
Clusters 3-6
Clusters 1 and 2
Preop
3 mo
6 mo
12 mo
E
p = 0.12
Postop Improvement in SNOT-22 Score
60
40
20
0
-20
Clusters1 and 2
Clusters 3-6
F
p = 0.04
% improvement in SNOT-22 Score
100
0
-100
-200
-300
Clusters 1 and 2
Clusters 3-6