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To the Editor:

The identification and characterization of chronic rhinosinusitis (CRS) inflammatory endotypes has advanced the understanding of CRS pathophysiology with potentially important clinical implications¹⁻³. Building upon these innovative studies, our group recently characterized CRS inflammatory endotypes in a North American population using cluster analysis of cytokines measured in sinonasal mucus^{4,5}. Cluster analysis of 147 adults with medically refractory CRS revealed phenotypic and inflammatory signatures unique to the different groupings. Certain clusters were characterized by low levels of most assayed cytokines, milder disease phenotypes with lower proportions of comorbid asthma and nasal polyps, and greater postoperative improvement in disease-specific quality of life (QoL) scores⁵. Conversely, other clusters demonstrated more severe disease and poor long-term response to surgical intervention. Despite the apparent clinical implications of these findings, the longitudinal stability of CRS endotypes is not yet known. The aim of this investigation was to assess the stability of previously validated inflammatory CRS endotypes over time and to determine the effect of surgical intervention on inflammatory burden and endotypic assignment.

We resampled twenty (10 CRSsNP, 10 CRSwNP) of the 147 subjects analysed in our initial study using the same minimally invasive approach⁴ during routine follow-up (Supporting Table S1). Mucus biomarkers have several advantages over surgically-obtained tissue in its minimally-invasive, yet comprehensive sampling of inflammatory mediators without being limited by potential variability in site-specific protein expression⁶. Repeat mucus collection was performed no sooner than 6 months after initial sampling (median = 15.5 months; range 10-41 months), the point following which few changes in postoperative disease-specific QoL are thought to occur and a suggested primary end point of CRS clinical studies⁷. As previously described, inflammatory burden in individual samples was measured using a multiplex assay that reflects various T_H1/T_H2/T_H17-associated cytokines⁵ (Table 1).

We first performed paired comparisons between matched samples to determine whether individual cytokine postoperative levels changed over time. The overall cohort was marked by significant increases in IL-7 ($p=.02$) and IFN- γ ($p=.003$) and significant reductions in IL-13 ($p=.01$) and RANTES ($p=.001$). Most changes were driven by patients with nasal polyps. Longitudinal evaluation of inflammatory burden in CRSwNP patients showed substantial reductions in IL-5 ($p=.01$), IL-13 ($p=.01$), and RANTES ($p=.01$), while CRSsNP patient were characterized by an increase in IFN- γ ($p=.02$).

We next sought to determine whether temporal changes in inflammatory biomarkers affect the endotypic assignments of each resampled subject. As previously reported, principal component analysis was performed on the entire data set to evaluate associations between immunologic variables^{4,5}, with a 5-factor solution explaining 67% of the variance (Supporting Table S2). Matched baseline and follow-up sampling of representative cytokines for each rotated factor are presented in Figure 1A. Using the factor scores calculated for each patient, hierarchical cluster analysis was again performed for all 147 patients repeated after replacing the original data for the 20 resampled patients with their repeat cytokine measurements (Figure 1B). Consistent with our prior study, analysis again converged on a five cluster model, validated using statistical modelling and bootstrapping. Alteration of the dataset with the resampled patients resulted in minor changes to dendrogram nodal patterns and subject allocation, but a similar architecture to the original analysis was maintained. The majority of patients (89%) who were not resampled were assigned to the same disease cluster as the original analysis, suggesting stability of the model and previously validated endotypes.

Conversely, 13 of 20 resampled subjects (65%) switched disease clusters (Supporting Table S3). There was a notable convergence of resampled subjects into cluster 5, which is characterized by mild-moderate disease severity, good olfactory function, and low tissue eosinophilia^{4,5}. This cluster is further defined by low overall inflammatory burden, but the highest levels of IL-7 and lowest levels of RANTES. This disproportionate reassignment of subjects to cluster 5 may derive from relatively consistent changes in these cytokines in the overall cohort and from a general reduction in inflammatory burden in postoperative patients. The likelihood of cluster switching was independent of demographic factors, symptom burden, and both polyp and asthma status (Supporting Table S4).

To our knowledge, this is the first study to evaluate the long-term stability of CRS inflammatory endotypes and following surgical intervention. Surgery in patients with nasal polyps appears to result in profound and long-term reductions in the T_H2-associated cytokines IL-5 and IL-13. These changes are comparable or greater to changes in T_H2 mediators observed in CRS patients after treatment with dupilumab⁸ and may reflect substantial changes in the underlying immune environment after surgical and medical management. In contrast, a majority of patients with or without polyps experienced a significant elevation in IFN- γ . Taken together, these changes in cytokine levels suggest surgical intervention may ultimately shunt inflammatory burden from a T_H2-

to T_H1 pattern. Collectively, our data suggests that surgery results in permanent alterations in CRS inflammatory burden that can change endotypic assignment. This observation has clinical relevance given that patients with a history of prior surgery accounted for approximately 30-60% of subjects in recent CRS endotyping studies²⁻⁵.

Limitations to the current study include its small sample size and variable follow-up. Future investigations with increased study subjects may more specifically characterize to what degree differences in the interval between surgery and resampling among patients may impact cluster analysis. Because all patients in the current study underwent endoscopic sinus surgery between initial and follow-up sampling, it is difficult to dissociate temporal effects and other factors from surgery itself. Additional work in this area would also benefit from a comparison group of recalcitrant, nonsurgical CRS patients and from a focused evaluation of potential associations between cytokine levels, cluster assignment, and long-term symptom burden.

Recent investigations have expanded understanding of CRS pathophysiology and biomarkers largely by improving CRS classification schemes and defining putative CRS endotypes¹. These methods have potential both in directing patients to optimal therapeutic approaches based on inflammatory profiles and in following response to treatment. Sampling of nasal mucus is minimally invasive, easily collected, and facilitates monitoring of inflammatory changes over time. While the current study analysed a comparatively small sample size, it suggests that endotypic assignments are not static and can potentially be impacted by clinical and environmental factors. Future prospective studies to evaluate endotype stability over time and in response to corticosteroids, immunomodulating therapy, and surgery are needed. Ultimately, incorporation of dynamic (cytokines, inflammatory biomarkers, etc.) and static (polyp/asthma status, sex, etc.) variables into CRS endotyping may improve stability and its predictive ability⁹.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Table 1. Cytokine and inflammatory mediator concentrations (pg/mL).

	CRS Phenotype						All (n=20)		
	CRSwNP (n=10)			CRSSNP (n=10)			Preoperative	Postoperative	P value
	Preoperative	Postoperative	P value	Preoperative	Postoperative	P value			
IL-1 β	120.8 (55.6-459.1)	174.0 (24.9-496.1)	.70	947.4 (121.1-4411)	177.2 (7.9-3985)	.32	175.4 (79.6-1404)	177.2 (10.3-637.1)	.65
IL-2	11.1 (2.2-47.8)	19.1 (2.2-34.2)	.91	2.6 (2.2-15.9)	15.2 (4.3-173.9)	.16	8.0 (2.2-27.7)	15.2 (2.4-38.2)	.25
IL-4	1.5 (0.3-3.0)	0.5 (0.3-1.9)	.28	0.3 (0.3-2.2)	1.2 (0.3-1.9)	.47	0.5 (0.3-2.3)	0.6 (0.3-1.7)	.68
IL-5	383.9 (67.1-474.9)	16.1 (2.7-275.3)	.01	1.4 (0.2-2.5)	1.4 (0.8-6.0)	.36	6.6 (1.1-385.7)	5.9 (1.1-55.8)	.08
IL-6	448.8 (72.8-1461)	596.6 (144.6-2792)	.77	218.0 (80.6-1045)	401.0 (98.1-2308)	.49	240.4 (82.3-1105)	462.7 (120.9-1972)	.45
IL-7	5.9 (2.8-18.3)	28.2 (13.7-73.3)	.03	14.6 (2.2-43.8)	34.9 (12.2-86.8)	.19	6.6 (2.9-21.6)	28.2 (13.9-77.8)	.02
IL-8	5817 (2506-14113)	38634 (27969-72604)	.08	45221 (8296-100717)	74844 (28813-101279)	.56	12236 (4229-46040)	47125 (28320-89654)	.11
IL-9	12.2 (0.6-29.6)	2.9 (0.6-8.9)	.46	1.6 (0.6-7.2)	3.6 (1.1-21.3)	.19	2.9 (0.6-18.5)	3.4 (0.8-10.7)	.71
IL-10	14.3 (3.7-27.1)	9.4 (6.0-19.8)	.63	6.4 (0.3-22.6)	13.1 (4.2-29.9)	.18	12.6 (1.0-23.1)	9.9 (5.6-20.4)	.74
IL-12	68.2 (6.9-91.8)	17.0 (9.1-50.1)	.11	86.5 (22.7-337.9)	42.0 (5.4-78.7)	.15	73.2 (13.8-104.6)	36.2 (7.2-55.7)	.07
IL-13	198.4 (64.0-388.0)	18.7 (11.1-85.0)	.01	12.1 (0.3-50.9)	9.1 (2.0-16.9)	.21	55.7 (4.3-211.0)	14.4 (5.0-23.0)	.01
IL-17A	2.8 (0.1-8.9)	3.2 (0.3-7.8)	.76	0.1 (0.1-5.5)	3.1 (1.5-6.9)	.74	1.6 (0.1-7.7)	3.1 (0.4-5.9)	.56
IL-21	75.7 (32.0-163.1)	182.0 (76.1-219.5)	.49	101.9 (6.9-259.2)	175.7 (73.6-325.5)	.99	75.7 (10.9-234.8)	175.7 (96.5-241.3)	.62
TNF- α	14.2 (3.0-30.1)	14.2 (7.7-40.1)	.80	5.4 (0.8-18.5)	11.9 (5.1-29.6)	.23	7.4 (1.4-18.5)	12.3 (6.1-38.1)	.35
IFN- γ	2.1 (0.7-4.7)	3.6 (2.0-9.5)	.06	0.5 (0.4-6.4)	4.6 (2.6-7.5)	.02	1.2 (0.4-4.7)	3.9 (2.6-7.5)	.003
Eotaxin	41.1 (8.3-83.5)	99.2 (18.5-253.0)	.49	27.1 (3.3-79.2)	63.3 (48.5-103.7)	.13	36.2 (7.1-79.9)	63.3 (35.0-176.9)	.11
RANTES	2240 (308.1-4855)	71.3 (24.1-278.9)	.01	495.4 (116.8-4929)	190.0 (103.4-308.7)	.11	1173 (192-4007)	162 (54.3-287.3)	.001

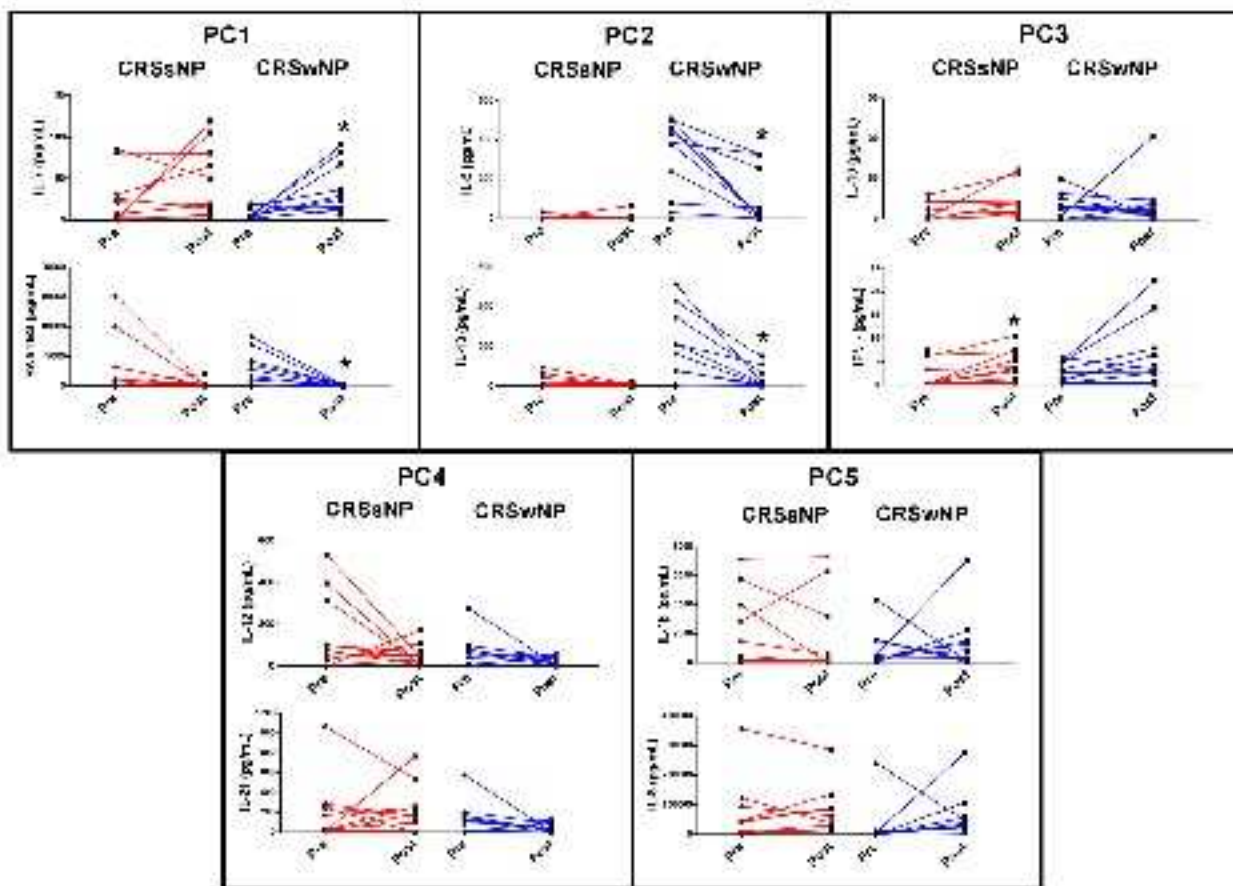
CRS, chronic rhinosinusitis; CRSSNP, chronic rhinosinusitis without nasal polyposis; CRSwNP, chronic rhinosinusitis with nasal polyposis; TNF, tumor necrosis factor

Data are represented as medians with interquartile ranges. Boldface text indicates a p value of less than .05.

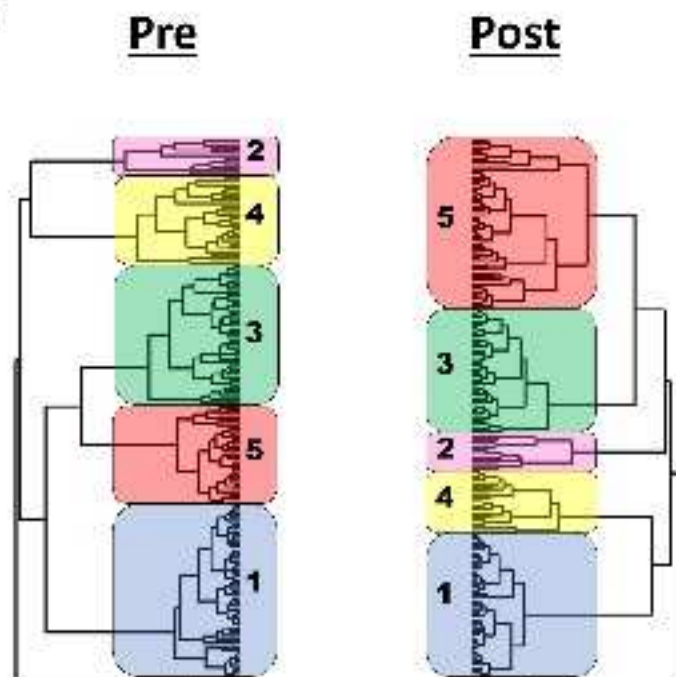
FIGURE LEGENDS

Figure 1. (A) Concentrations of representative inflammatory mediators from each of the five rotated factors at baseline and after repeat sampling. **(B)** Side-by-side comparison of original baseline dendrogram derived from hierarchical cluster analysis from initial sampling (left) and dendrogram after repeat sampling of 20 subjects (right).

A



B



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METHODS

Study design and patient population

University Institutional Review Board approval was obtained. Patients presented to the Vanderbilt Asthma, Sinus, and Allergy Program and Otolaryngology Clinic at the Vanderbilt Bill Wilkerson Center for routine follow-up. Patients meeting inclusion criteria who had previously been enrolled in CRS endotyping studies published by our group were identified without respect to disease phenotype or disease control. All patients had previously undergone functional endoscopic sinus surgery for medically refractory CRS, diagnosed according to established guidelines^{S1, S2}. Included patients had diffuse, bilateral CRS. Patients with disease isolated to the osteomeatal complex or secondary to odontogenic disease and fungus balls were excluded. Patients were also excluded in the presence of comorbid cystic fibrosis, autoimmune or granulomatous disease, and treatment with systemic steroids or antibiotics within 4 weeks of mucus collection. The extent of surgery was similar in all patients, with approximately 80% undergoing pan-endoscopic sinus surgery. Patients remained on their nasal regimens and oral antihistamine and leukotriene medications, consistent with the original sampling conditions. No samples were obtained in the setting of an active infection or asthma exacerbation.

Smoking status and comorbid asthma, aspirin-exacerbated respiratory disease (AERD) and allergic rhinitis were recorded. Asthma was diagnosed based on metacholine challenge, pulmonary function testing and/or by a pulmonologist. AERD was designated in cases of concomitant asthma, nasal polyposis, and prior positive aspirin challenge testing or ≥ 2 episodes of respiratory reactions to aspirin or nonsteroidal anti-inflammatory drugs. Allergic rhinitis was diagnosed by positive serum or intradermal allergen tests.

Patients previously underwent high-resolution computed tomography (CT) scan of the paranasal sinuses within 3 months of surgery, and CT scans were independently scored to using the Lund-Mackay system^{S4}. Subjects enrolled in the previously described study^{S4} had also completed the 40-item Smell Identification Test (SIT) immediately before 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^{S5}. Raw scores were adjusted for patient age and sex by subtracting the mean normative age- and sex-appropriate SIT score from the total SIT score for each subject. Thus, a negative-adjusted SIT score represents reduced sense of smell compared with the mean for that subject's age and sex. Normative SIT scores were extracted from the Smell Identification Test Administration Manual^{S6}. Patient-reported symptom severity was measured using the SNOT-22 before and after surgery^{S7}. Postoperative surveys completed at 3-, 6-, 12- and 24-months were averaged together to give a single, composite postoperative score for each patient. Higher scores reflect higher subjective disease burden.

Sample collection and cytokine assay

As previously described, 9 x 24 mm polyurethane sponges (Summit Medical; St. Paul, MN) were placed in the middle meatus or ethmoid cavities of subjects under endoscopic guidance^{S3, S4, S8}. This approach has been previously validated and has advantages over other methods for mucus collection, including standardization between subjects and avoidance of specimen dilution^{S9-11}. Sponges were left in place for 5 minutes and then transferred to sterile microcentrifuge tubes for immediate processing. Specimens were centrifuged at 14,000 g for 10 minutes to elute mucus in a microporous centrifugal filter device (Millipore Sigma; Billerica, MA). Samples were then gently vortexed and centrifuged for an additional 5 minutes to remove cellular debris. Supernatants were removed, placed into a new microcentrifuge tube, and stored at -80°C for later analysis.

Cytokine assays were performed with a multiplex cytokine bead assay according to the manufacturer's protocol (BD Biosciences; Franklin Lakes, NJ). 50 µL of mucus was incubated with 50 µL of mixed capture beads for each inflammatory mediator for 1 hour. 17 cytokines and inflammatory mediators were analysed, excluding only those with overlapping detection wavelengths and most growth factors and chemokines. 50 µL of mixed detection reagent was added, and the specimens were incubated for another 2 hours. Next, 1 mL of wash buffer was added, and samples were centrifuged at 200 g for 5 minutes. The supernatant was discarded, and the beads were resuspended in 300 µL of wash buffer and

analysed on an LSR Fortessa flow cytometer (BD Biosciences; Franklin Lakes, NJ). Data were analysed with BD FCAP Array Software, version 3.0 (BD Biosciences; Franklin Lakes, NJ).

Statistics

Data from the twenty resampled patients were inserted in place of the same patients' preoperative data. Principal component analysis and hierarchical clustering were performed as previously reported, with the following minor variations^{S3, S4}. Descriptive statistics and frequency distributions were examined for each biological variable, and all were positively skewed. To normalize data for subsequent analysis, values were natural log transformed, eliminating or significantly reducing skewing for all variables. A principal-component factor analysis with varimax rotation was then performed on the transformed biological variables. The appropriate number of factors was selected by analysis of the Scree plot. Each factor had an eigenvalue > 1.0. The regression method was then used to calculate a factor score for each subject in each of the 5 factors.

Hierarchical cluster analysis was then performed using the Ward method on squared Euclidian distances using the 5 factor scores. The Ward method determines which clusters can be merged by comparing dissimilarity between subjects and by minimizing total within-cluster variance^{S12}. This method can simplify a complex dataset with multiple subjects and variables and finds natural groupings of subjects based on input variables. The hierarchical structure and taxonomic relationships between subjects was visualized using a dendrogram (Figure 1A).

The appropriate number of clusters (k) was selected using the Elbow method. This approach calculates the total within sum of squared error for between 1 and 10 clusters. k is determined by identifying the break point, where the addition of subsequent clusters does not substantially alter the sum of squared error. Cluster stability was verified by bootstrap analysis. The data were randomly resampled 1000 times to ensure that the clustering results were stable and not unique to the original dataset. Cluster stability ranged between 0.6 and 0.9, where values closer to 1 indicate more stable clusters. Baseline and

resampled cytokine levels were compared using the Wilcoxon matched-pairs signed rank test. A *P* value <.05 was considered statistically significant for all comparisons. Data management and analysis was conducted with R software (version 3.4; R Foundation for Statistical Computing, Vienna, Austria) and Prism 6 software (GraphPad, La Jolla, CA).

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120 **Table S1.** Patient clinical and phenotypic characteristics.

	Patient Variables																
	Age	Gender	Race	Polyposis	Prior	AERD	Asthma	AR	Current	NCS	NAH	OAH	LTR	Preoperative	Preoperative	Preoperative	Postoperative
					Surgery				Smoker	use	use	use	use	CT score	SIT score	SNOT-22	SNOT-22
	53.8	10 (50)		10 (50)	12 (60)	6 (30)	16 (80)	14 (70)	1 (5)	17 (85)	4 (20)	10 (50)	9 (45)	17.8 (6.0 - 20.3)	-17.5 (-25.9 to -2.0)	44.31 ± 19.53	20.66 ± 17.91
Subject																	
1	66	F	C	-	Y, 1	-	+	+	-	+	-	+	-	18.5	-25	27	19
2	61	F	C	-	Y, 1	-	+	-	-	+	+	-	-	7	1	NR	24
3	69	F	C	-	N	-	+	+	-	-	-	+	-	7	-5	57	56
4	75	M	C	-	N	-	-	+	-	i	-	-	-	6	NR	NR	24
5	39	F	AA	-	N	-	-	+	-	-	-	+	-	11	-7	76	28.5
6	68	M	C	-	Y, 7	-	+	-	-	-	-	-	-	11	-1	NR	9
7	62	F	C	-	N	-	+	+	-	+	-	+	+	19	-26	20	4
8	65	F	C	-	Y, 1	-	+	+	-	+	+	+	-	10	NR	67	53.7
9	48	M	C	-	N	-	-	+	-	+	-	-	-	20	-5	NR	16
10	73	M	C	-	N	-	+	-	-	+	+	-	-	NR	0	34	14
11	63	F	C	+	Y, 2	+	+	+	-	+	+	-	+	22	-26	35	12
12	41	F	C	+	Y, 2	+	+	+	-	+	-	+	+	17.5	-25.5	NR	3
13	27	F	C	+	Y, 1	+	+	+	-	+	-	+	-	18	-23	27	3
14	44	M	C	+	Y, 2	-	-	+	-	i	-	+	+	16	NR	NR	7.5
15	45	M	C	+	Y, 3	+	+	+	-	+	-	-	+	20	-30	54	20
16	36	M	C	+	Y, 1	+	+	-	+	+	-	-	+	NR	-30.5	52	13
17	36	M	C	+	Y, 3	-	+	+	-	+	-	+	+	23.5	-12	65	43.5
18	50	M	C	+	Y, 2	+	+	-	-	+	-	-	+	21	NR	NR	55
19	57	F	C	+	N	-	+	-	-	+	-	-	-	24	-24.5	48	5
20	50	M	H	+	N	-	+	+	-	+	-	+	+	12	0	14	3

121 AA, African-American; AR, allergic rhinitis; AERD, aspirin exacerbated respiratory disease; BMI, body mass index; C, Caucasian; CRS, chronic rhinosinusitis; H, Hispanic; I, intermittent; LTR, oral
 122 antileukotriene; NAH, nasal antihistamine; NCS, nasal corticosteroid; NR, not recorded; OAH, oral antihistamine

123 Values are presented as frequencies (percentages), means ± SD or medians with interquartile ranges, depending on the normalcy of data.

Table S2. Loading of biological variables after principal component analysis.

Variable	Rotated Factor				
	1	2	3	4	5
IL-6	0.537				0.503
IL-7	0.737				
Eotaxin	0.671				
IL-5		0.912			
IL-9		0.738			
IL-13		0.891			
IL-10			0.898		
IFN- γ			0.904		
IL-12				0.705	
IL-21				0.814	
IL-1 β					0.834
IL-4					0.607
IL-8					0.721
IL-17A					0.517
TNF- α					0.733

Values for biological variables were transformed to achieve normalcy and then analysed with principal-component analysis with varimax rotation. Variables with a loading of >0.5 were retained. Data for the first 5 factors are shown on the basis of analysis of the plotted Eigenvalues for each component. The 5-factor solution explained 67.5% of the collective data variance

129 **Table S3.** Cluster changes among resampled patients.

n (%)	Cluster		Change
	Preoperative	Postoperative	
Subject			13 (65)
1	5	5	n
2	1	5	y
3	1	5	y
4	5	5	n
5	3	3	n
6	3	5	y
7	5	5	n
8	1	2	y
9	3	5	y
10	1	5	y
11	4	3	y
12	4	4	n
13	3	5	y
14	4	4	n
15	4	5	y
16	1	5	y
17	1	5	y
18	4	4	n
19	4	3	y
20	4	5	y

130

131

Table S4. Clinical and demographic factors based on cluster stability.

	Cluster			All
	<i>Stable</i> (n=7)	<i>Changed</i> (n=13)	<i>P value</i>	(n=20)
Age (y)				53.8
Gender (female)	4 (57)	6 (46)	>.99	10 (50)
Race (Caucasian)	6 (86)	11 (85)	>.99	18 (90)
Prior Surgery	4 (57)	9 (69)	.65	12 (60)
Current Smoker	0 (0)	1 (8)	>.99	1 (5)
CRSwNP	3 (43)	7 (54)	>.99	10 (50)
Asthma	4 (57)	12 (92)	.10	16 (80)
AERD	2 (29)	4 (31)	>.99	6 (30)
AR	6 (86)	8 (62)	.35	14 (70)
NCS use	6 (86)	11 (85)	>.99	17 (85)
NAH use	0 (0)	4 (31)	.25	4 (20)
OAH use	5 (71)	5 (38)	.35	10 (50)
LTR use	4 (57)	5 (38)	.64	9 (45)
SIT score	-25.3 (-25.9 to -11.5)	-8.5 (-25.6 to -0.3)	.27	-17.5 (-25.9 to -2.0)
CT score	17.5 (11-19)	18.0 (10 - 22)	.67	17.8 (6.0 - 20.3)
Preoperative SNOT-22	45.3 ± 17.2	41 ± 30.5	.83	44.3 ± 19.5
Postoperative SNOT-22	20.1 ± 18.3	20.9 ± 18.4	.93	20.7 ± 17.9

AA, African-American; AR, allergic rhinitis; AERD, aspirin exacerbated respiratory disease; BMI, body mass index; C, Caucasian; CRS, chronic rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyposis; H, Hispanic; I, intermittent; LTR, antileukotriene; NAH, nasal antihistamine; NCS, nasal corticosteroid; OAH, oral antihistamine

Values are presented as frequencies (percentages), means ± SD or medians with interquartile ranges, depending on the normalcy of data.