

Assessing tissue transcription biomarkers of chronic rhinosinusitis: a comparison of sampling methodologies

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Background: Chronic rhinosinusitis (CRS) is a spectrum of complex inflammatory conditions of the sinonasal mucosa. Identification of biomarkers that enable classification and improved delineation among CRS endotypes is of increasing interest. However, the extent to which less invasive sampling methods identify genuine tissue inflammatory patterns is not well understood. The aim of this study was to investigate mucosal swab and cytobrush sampling as less invasive proxies for tissue transcription levels of putative biomarkers of CRS.

Methods: Expression levels of 21 biomarkers of interest were assessed via custom TaqMan array cards from mucosal biopsy, cytobrush, and swab samples, in 32 patients with CRS. Reported expression levels were compared between each of the 3 sample types within each patient.

Results: Reported transcription levels from swab samples for *IL33*, *MUC5AC*, *IL1RN*, *CXCL8* (IL-8), *TNF*, *IFNG*, *IL5*, *OSM*, *IL1A*, and *IL17C*, and cytobrush levels for *IL33*, *MUC5AC*, *IL5RA*, *IL1RN*, *CXCL8* (IL-8), and *IL5* were significantly different to tissue levels from matched biopsy samples.

Conclusion: Reported expression via swab and cytobrush sampling differed from patterns observed in matched tissue for 10 of 21 and 6 of 21 markers, respectively. Non-biopsy-based studies for these particular markers may therefore not adequately represent tissue inflammatory processes and should be interpreted with caution. Cytobrush samples largely tracked tissue patterns for the remaining target biomarkers. In these cases, cytobrush sampling appears to adequately reflect tissue patterns for several putative biomarkers of CRS, supporting their use in clinical and research settings as a less-invasive proxy for the assessment of mucosal tissue inflammatory transcription patterns. © 2020 ARS-AAOA, LLC.

Key Words:

chronic rhinosinusitis; endotypes; inflammatory disease; mucosa; biomarkers; cytokines

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Chronic rhinosinusitis (CRS) is a common and debilitating chronic inflammatory condition of the sinonasal mucosa.^{1,2} CRS is generally differentiated based on associated polyposis (CRS without nasal polyps [CRSsNP], and CRS with nasal polyps [CRSwNP]).¹ However, additional

layers of heterogeneity within these groups are also evident, and CRS is increasingly thought of as an array of distinct conditions.³⁻¹³ Distinctions in underlying inflammatory processes have been identified based on different patient populations (in particular, European and North American compared with Asian patients), associated cystic fibrosis, and markers of eosinophilia.^{8,9,14-19} Mixed inflammatory types (T helper (Th)-1, Th-2, and/or Th-17-type markers elevated in parallel) have also been observed in some patients.^{6,13}

Inadequate delineation of subtypes of CRS is likely to have hindered previous efforts to identify specific disease mechanisms, and to make informed treatment decisions. Identification of markers that could be used for diagnosis of CRS endotypes offers a promising step toward personalized treatment approaches better tailored to the specific mechanisms underlying disease processes in each individual patient. As such, a great deal of recent work has focused on improved delineation among

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different CRS endotypes.^{3-6,8,11-13,17,18,20} Numerous potential markers of CRS and/or CRS endotypes have been identified, including interleukin (IL)-2, IL-4, IL-5, IL-6, IL-6-receptor, IL-8, IL-10, IL-12, IL-13, IL-17, IL-17-receptor-beta, IL-25, IL-33, interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, transforming growth factor (TGF)-beta, oncostatin M (OSM), C-C motif chemokine ligand 18 (CCL18), eotaxin-2, eotaxin-3, Charcot-Leyden Crystal (CLC), eosinophil cationic protein (ECP), immunoglobulin E (IgE), IgD, mucin 5AC (MUC5AC), and MUC5B.^{3,6,8,9,13-16,18,21-37} Importantly, however, a variety of sample types have been examined, including tissue biopsies (nasal polyp, ethmoid, inferior turbinate, uncinate, and/or sinus mucosal biopsies),^{6,13,17-19,22-24,26-29,34-36,38-48} cytobrushes,⁴⁹ and mucus and nasal secretion samples.^{25,50-54} The comparability of biomarker data reported for each of the different sample types is unclear. For example, it is unknown whether sampling at or above the mucosal epithelium (such as cytobrush, swab, or mucus sampling) accurately reflects actual transcription patterns within the tissue below.

Identifying less invasive sampling methods that reflect tissue inflammatory processes is essential for implementing translational applications of CRS endotyping in the clinic setting. Tissue biopsies are invasive, and routine tissue sampling in outpatient clinics is technically challenging and may be uncomfortable for patients. CRS endotyping via biopsies may therefore be limited to intraoperative collection to guide postsurgical treatment. The identification of suitable, less invasive proxies would enable endotyping of patients in clinic and could be implemented in standard medical management practices to better guide individualized treatment options. Similarly, noninvasive sampling would allow for better consistency of sampling sites across all patient groups (including controls), as well as time-series studies that are otherwise not feasible due to the invasive nature of tissue biopsy collection.

The aim of this study was to investigate the accuracy of mucosal swab and cytobrush sampling as less invasive proxies for tissue transcription levels of biomarkers of putative endotypes of CRS. Twenty-one biomarkers of interest were assessed via mucosal biopsy, cytobrush, and swab sampling, in 32 patients with CRS.

Patients and methods

Patient recruitment and sample collection

Thirty-two patients who had elected to undergo functional endoscopic sinus surgery (FESS) for CRS were recruited, including 18 with CRSsNP and 14 with CRSwNP. CRS diagnosis was as per the European Position Paper on Rhinosinusitis (EPOS2012) guidelines.¹ Exclusion criteria included sinonasal vasculitis, immunodeficiency, and patient <18 years of age. Ethical approval was obtained from the New Zealand Health and Disability Ethics Commit-

TABLE 1. Patient demographics*

Demographic	CRSsNP (n = 18)	CRSwNP (n = 14)
Age (years), median (range)	44 (20–74)	38 (19–63)
European, n (%)	17/18 (94)	12/14 (86)
Gender, female, n (%)	8/18 (44)	4/14 (29)
Smoker, n (%)	1/17 (6)	0/14 (0)
Asthma, n (%)	4/18 (22)	4/14 (29)
Aspirin sensitivity, n (%)	0/18 (0)	0/14 (0)
Antibiotics, n (%) ^a	1/18 (6)	0/14 (0)
Corticosteroids, n (%) ^a	1/18 (6)	0/14 (0)
SNOT-22 symptom score, median (range)	43 (5–106)	48 (7–79)
Lund-Mackay, median (range)	11.5 (5–23)	13 (6–21)

*Continuous variables are presented as median (range). Categorical variables are presented as proportion yes/total, except gender, which is presented as the proportion of female participants.

^aWhether patients had taken antibiotics or corticosteroids in the 4 weeks prior to surgery.

CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps; SNOT-22 = 22-item Sino-Nasal Outcome Test.

tee (NZX/08/12/126), and written informed consent was obtained from all participants.

At the time of surgery, clinical severity scores (SNOT-22⁵⁵ and Lund-Mackay), and patient demographics data were collected (including age, gender, ethnicity, concomitant asthma and/or aspirin sensitivity, smoking status, and whether the patients had taken antibiotics or corticosteroids in the 4 weeks prior to surgery) (Table 1). Samples were collected intraoperatively under endoscopic guidance prior to the administration of topical vasoconstrictor solutions. The following samples were collected for each patient for this study: 1 pair of swab samples (sterile, rayon-tipped; Copan Diagnostics, Inc., Murrieta, CA) from the middle meatus; 1 cytobrush sample (ConMed, Utica, NY; #CM-149) from the middle meatus; and a mucosal tissue biopsy from the bulla ethmoidalis. All samples for each patient were collected from the same side of the sinonasal tract. Samples were placed immediately into sterile 1.5-mL tubes together with RNAlater (Life Technologies, Auckland, New Zealand), transported to the laboratory on ice, and stored at –20°C until further processing.

Sample processing

RNA was extracted from all samples using the AllPrep DNA/RNA Isolation kit (Qiagen, Hilden, Germany). Samples were placed in sterile Lysing Matrix E bead tubes (MP Biomedicals, Sydney, Australia) together with 600 µL RLT lysis buffer (including 10 µL/mL β-mercaptoethanol, as recommended), then cells were ruptured in a Tissue Lyser II (Qiagen) for 2 × 40 seconds at 25 m/second. Following centrifugation, the supernatant was transferred to the supplied spin columns and processed as per the manufacturer's

instructions. Following extraction, RNA was deoxyribonuclease (DNase)-treated using amplification grade DNaseI (Life Technologies), and the successful removal of residual DNA confirmed via β -actin polymerase chain reaction (PCR). RNA was reverse-transcribed to complementary DNA (cDNA) using Superscript IV VILO Master Mix (Life Technologies) as per the manufacturer's instructions, and quality and quantity assessed via NanoPhotometer (N60; Implen GmbH, München, Germany).

Biomarker expression: TaqMan array cards

Custom TaqMan quantitative PCR (qPCR) array cards (384, format 24) (Life Technologies) were used to assess gene expression levels of biomarkers of interest. The following 21 target genes were selected based on previously implicated biomarkers in CRS^{6,8,13,14,17,18,23,25–29,32,35,36,56,57}: IL-1-alpha (*IL1A*), IL-1 receptor antagonist (*IL1RN*), *IL4*, *IL5*, IL-5 receptor (*IL5RA*), *IL6*, *OSM*, IL-8 (*CXCL8*), *IL10*, IL-10 receptor alpha (*IL10RA*), *IL13*, *IL17F*, *IL17C*, *IL22*, *IL33*, IFN- γ (*IFNG*), *TNF*, *MUC5AC*, myeloperoxidase (*MPO*), eosinophilic cationic protein (*RNASE3*), and Fc fragment of IgE receptor 1a (*FCER1A*). *RNA18S*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) were also included as normalization controls. All genes were assessed in duplicate for each sample and averaged for further analysis.

Samples were prepared and array cards processed using a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Auckland, New Zealand), as per the manufacturer's instructions. In brief, 100 ng of cDNA was added to TaqMan Fast Advanced Master Mix (Life Technologies) and loaded into the sample ports of the array cards. Cards were briefly centrifuged, and then run under the following conditions: 50°C (2 minutes), 92°C (10 minutes), and 40 $\times$ cycles of 95°C (1 second), 60°C (20 seconds). To ensure comparability between samples run on different array cards, the cycle threshold (CT) level was held consistent for each gene of interest across samples (*CXCL8*, *IFNG*, *IL10*, *IL5RA*, and *RNASE3* = 0.3; *IL4* = 0.1; all remaining targets = 0.2), and raw data exported for further processing.

Data processing

Normalization

For each sample, the geometric mean of the 3 house-keeping genes (*RNA18S*, *GAPDH*, and *HPRT1*) was calculated and then used to generate normalized CT values (Δ CT) for each target gene of interest.⁵⁸ Δ CT values have an inverse relationship with expression levels, with lower Δ CT representing higher expression.

Overall sample type comparisons

To compare if observed transcript levels differed among sample types across the cohort as a whole, Δ CT values were assessed via ANOVA, with Benjamini and Hochberg

false discovery rate (FDR) adjustment. Post-hoc pairwise *t*-tests (with FDR adjustment for multiple comparisons) were conducted comparing between each of the 3 sample types (tissue, cytobrush, and swabs) for all variables with significant ANOVA FDR adjusted *p*-values.

Inpatient sample type comparisons

Fold differences in gene expression were compared between each of the sample types (tissue biopsy, cytobrush, and swabs) within each individual patient. For each gene (and each individual patient), tissue biopsy data were treated as baseline, and the difference from the other sample types calculated (comparative CT [Δ CT]) (swab vs tissue, and cytobrush vs tissue), generating swab and cytobrush Δ CT values for each patient for each target gene. All statistics were conducted and figures generated in R (v 3.6.2; R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>).⁵⁹ Swab and cytobrush Δ CT values were log-transformed (base 2) and assessed via 1-sided *t* tests, testing if the means differed from 0 (ie, differing from tissue "baseline" levels). Differences between swab and cytobrush data were compared via Welch 2-sample *t* tests.

To examine whether CRS phenotype might influence observed differences in the quantification of gene expression between each of the sample types, comparisons were also conducted based on clinical phenotype grouping (CRSSNP and CRSwNP).

Results

Overall sample type comparisons

The most highly expressed genes (regardless of sampling methodology) included *IL33*, *MUC5AC*, *IL5RA*, *FCER1A*, *IL1RN*, *CXCL8*, and *IL10RA* (Fig. 1; Supplementary Table 1).

Swab and cytobrush samples reported significantly higher expression levels (represented by lower Δ CT values) than from tissue biopsy for *MUC5AC* ($p < 0.001$), *IL1RN* ($p < 0.00001$), *CXCL8* ($p < 0.04$), and *IL17C* ($p < 0.04$), and significantly lower levels than tissue for *IL33* ($p < 0.001$) (Fig. 1). Reported *IL5RA* transcript level was significantly lower (reflected by higher Δ CT values) in the cytobrush samples than tissue or swab samples ($p < 0.00001$), and *MUC5AC* significantly lower in cytobrush sample data than that of swabs. Reported transcript levels were significantly higher in swab samples compared to tissue for *TNF* ($p = 0.010$) and *IL1A* ($p = 0.002$). Few significant differences in expression level were observed between CRSSNP and CRSwNP patients in overall sample type comparisons (Supplementary Fig. 1; Supplementary Table 2).

Inpatient sample type comparisons

Fold difference ($2^{\Delta\Delta\text{CT}}$) between observations made in tissue were compared to swab samples and cytobrush samples within each patient (Fig. 2). Median observed target gene

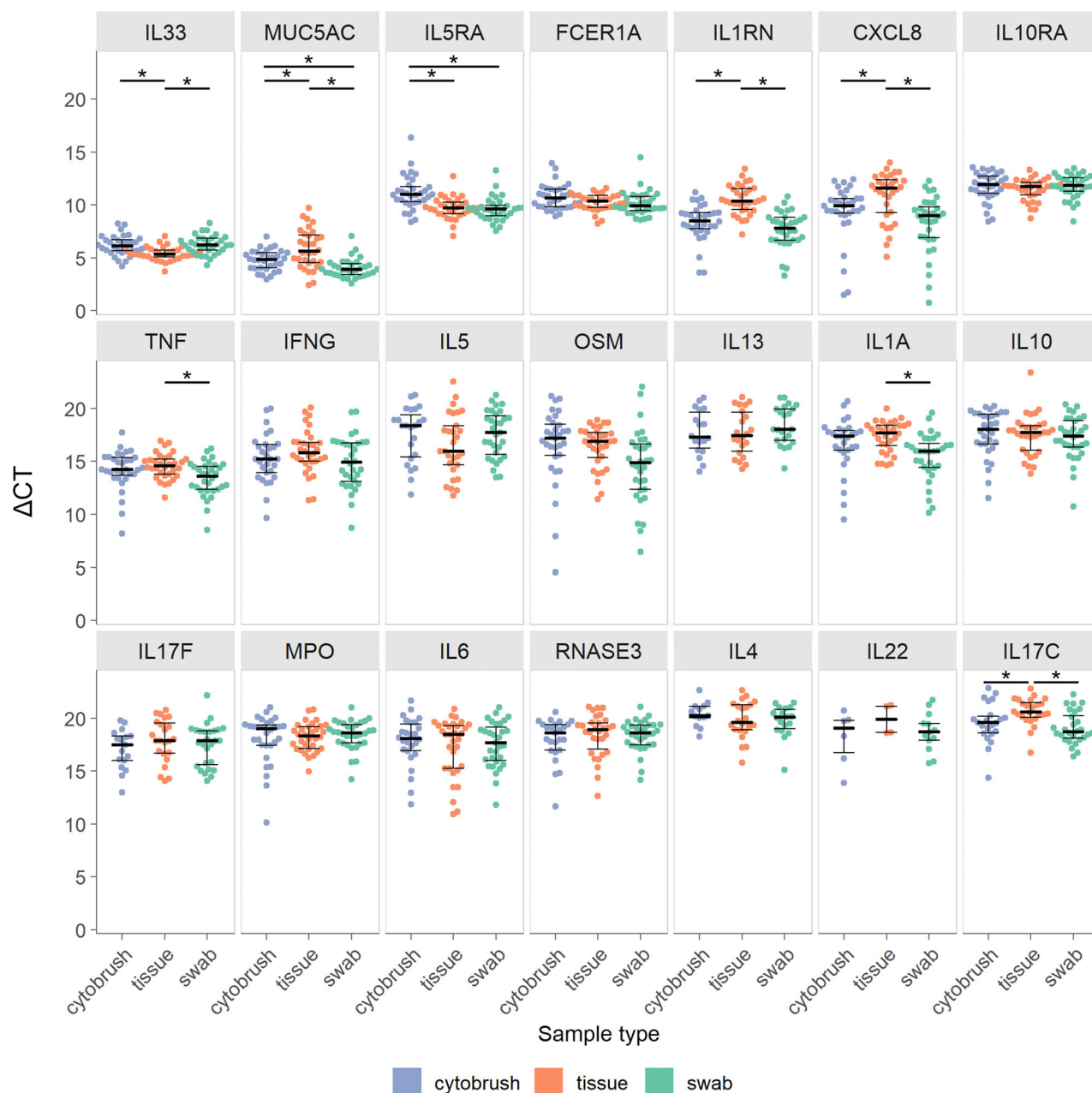


FIGURE 1. Δ CT values for 21 target biomarkers in 32 CRS patients. Markers were normalized based on the geometric mean of the expression of 3 additional genes: *RNA18S*, *GAPDH*, and *HPRT1*. Samples are split by the 3 sample types compared: mucosal cytobrush, tissue biopsy, and swab sampling. Biomarkers are presented in order of highest to lowest expressed genes across tissue samples (lower Δ CT values represent higher reported expression levels). Cross bars represent median and interquartile range. *Significantly different in pairwise comparisons (comparing between the pair joined by the significance bar tips) (FDR adjusted p value $\alpha = 0.05$). Δ CT = normalized cycle threshold; CRS = chronic rhinosinusitis; FDR = false discovery rate.

expression levels from cytobrush and swab samples were significantly 2-fold to 7-fold higher than tissue samples within the same patient for *MUC5AC* ($p < 0.01$), *IL1RN* ($p < 0.001$), *CXCL8* ($p < 0.01$), and significantly lower for *IL33* ($p < 0.001$), and *IL5* ($p < 0.05$) (Table 2; Supplementary Table 3). Swab sample data were also significantly elevated for *TNF*, *IFNG*, *OSM*, *IL1A*, and *IL17C* ($p < 0.01$), whereas cytobrush data were concordant with tissue data in these cases. *IL5RA* cytobrush data were significantly different to tissue data ($p < 0.001$), whereas the swab and tissue data were concordant in this case. In gen-

eral, cytobrush samples, and to a lesser extent swab samples, largely tracked the patterns observed in tissue samples for the remaining target genes.

Once data were split by CRS phenotype (CRSsNP or CRSwNP), the CRSsNP group appeared to be driving the observed inpatient sample type differences for *IL33*, *IL5RA*, *FCER1A*, *IFNG*, *IL5*, *OSM*, and *IL17C* ($p < 0.05$) (Supplementary Fig. 2; Supplementary Table 4). CRSwNP patient cytobrush and swab samples were not significantly different from tissue in these cases.

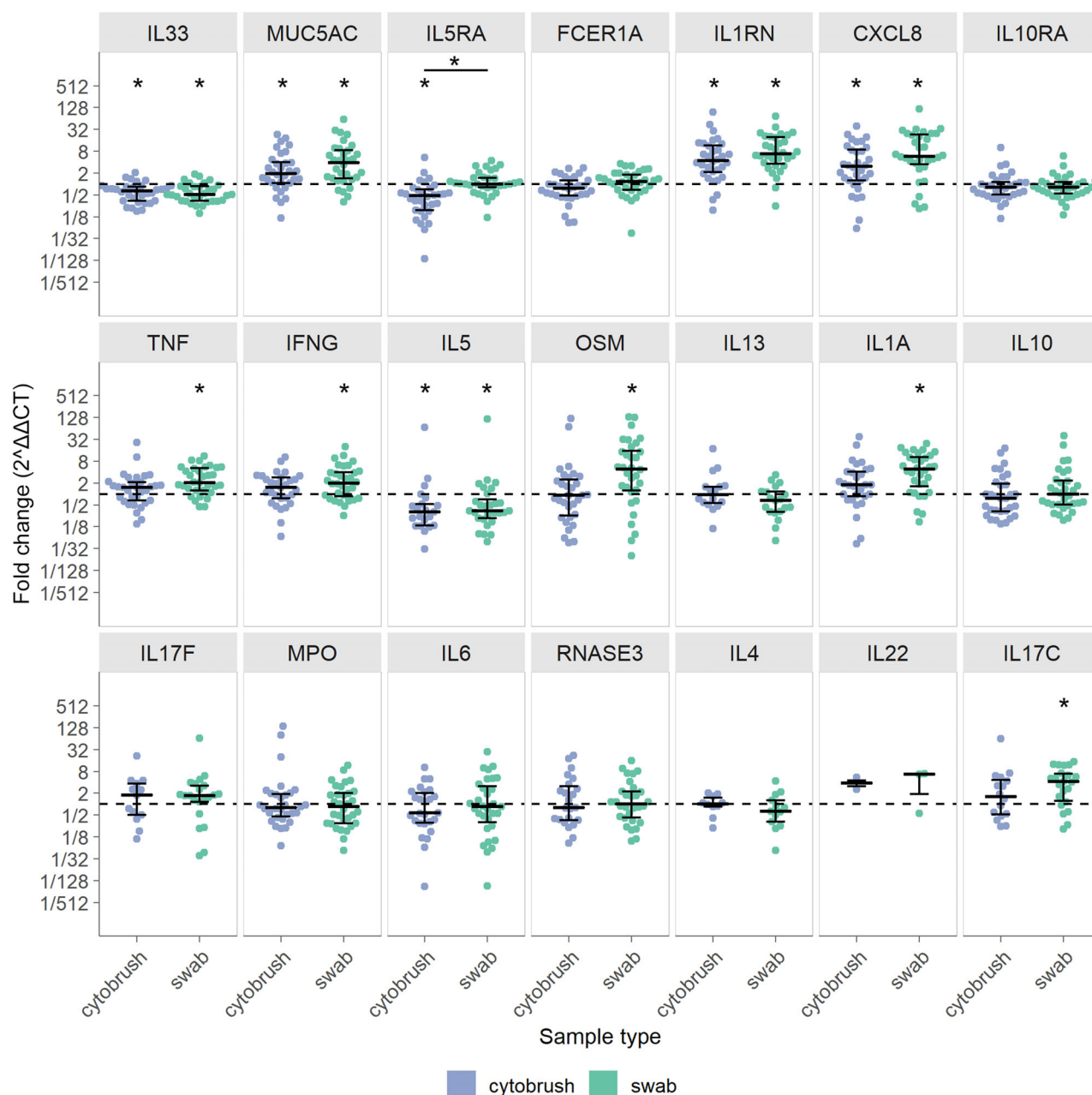


FIGURE 2. Intrapatient fold differences ($\Delta\Delta CT$) in biomarker expression reported by each sample type. Log₂ fold differences ($2^{\Delta\Delta CT}$) of cytobrush and swab samples compared with tissue biopsies (with reported expression of tissue biopsies taken as baseline). Cross bars represent median and interquartile range. Values >1 represent elevated reported expression, and <1 represent reduced reported expression, compared with the tissue sample from the same patient. *Significantly different to reported tissue expression in pairwise comparisons. *Significantly different in pairwise comparisons between the cytobrush and swab samples (FDR adjusted p value $\alpha = 0.05$). $\Delta\Delta CT$ = comparative cycle threshold; FDR = false discovery rate.

Discussion

There is currently a great deal of interest in identifying biomarkers that enable the classification and identification of CRS endotypes. The extent to which less invasive sampling methods adequately identify genuine tissue inflammatory patterns is not well understood. This study compared 3 common sampling methods, namely tissue biopsies, cytobrush samples, and swab samples. Twenty-one biomarkers of interest in CRS were assessed, and differences in the results reported by each sampling method investigated.

Biomarker expression levels in this cohort

Of the 21 markers assessed, the highest expression levels for all sample types (swabs, cytobrush, or tissue) were observed for *MUC5AC*, the cytokines *IL33* and *IL-8* (*CXCL8*), receptors for *IL-5* (*IL5RA*), *IL-10* (*IL10RA*), and *IgE* (*FCER1A*), and *IL-1* receptor antagonist (*IL1RN*). *MUC5AC*, *IL-33*, and *IL-8* have been identified as biomarkers of CRS or CRS endotypes.^{13,21,36,60-64} *IL-5*, *IL-10*, and *IgE* have also been implicated as important in CRS (the receptors for which were highly expressed in this study).^{6,26,36}

TABLE 2. Inpatient gene expression fold differences between sample type*

Gene	Cytobrush (n = 32)	Swab (n = 32)
IL33	0.65 (0.18–2.06) ^a	0.51 (0.16–1.89) ^a
MUC5AC	1.97 (0.12–23.36) ^a	3.89 (0.32–59.76) ^a
IL5RA	0.47 (0.01–5.42) ^{a,b}	1.01 (0.12–4.45) ^b
FCER1A	0.78 (0.09–2.74)	1.18 (0.04–3.63)
IL1RN	4.5 (0.19–96.03) ^a	6.72 (0.25–73.91) ^a
CXCL8	3.06 (0.06–39.6) ^a	5.86 (0.21–116.67) ^a
IL10RA	0.81 (0.11–10.02)	0.8 (0.14–5.92)
TNF	1.54 (0.15–26.59)	2.08 (0.45–10.98) ^a
IFNG	1.52 (0.07–10.38)	2.01 (0.26–20.41) ^a
IL5	0.33 (0.03–68.47) ^a	0.34 (0.05–116.93) ^a
OSM	0.91 (0.05–120.52)	4.92 (0.02–131.5) ^a
IL13	1 (0.11–17.84)	0.67 (0.05–3.38)
IL1A	1.77 (0.04–38.23)	4.85 (0.17–24.75) ^a
IL10	0.77 (0.15–18.22)	0.98 (0.19–40.35)
IL17F	1.8 (0.11–21.14)	1.74 (0.04–64.91)
MPO	0.81 (0.07–139.19)	0.88 (0.05–11.95)
IL6	0.57 (0.01–10.26)	0.85 (0.01–27.57)
RNASE3	0.79 (0.08–21.71)	1.02 (0.1–15.62)
IL4	1.03 (0.22–2.06)	0.65 (0.05–4.3)
IL22	4.03 (2.56–5.5)	6.66 (0.55–6.82)
IL17C	1.64 (0.24–63.91)	4.16 (0.21–14.73) ^a

*Data reflect inpatient $\Delta\Delta\text{CT}$ comparisons (cytobrush vs tissue, or swab vs tissue), and are reported as median $\Delta\Delta\text{CT}$ (range).

^aSignificantly different to tissue sample from the same patient (tested via 1-sample t test with FDR calculation; $\alpha = 0.05$).

^bSignificantly different between cytobrush and swab samples (tested via Welch 2-sample t test with FDR calculation; $\alpha = 0.05$).

$\Delta\Delta\text{CT}$ = comparative cycle threshold; FDR = false discovery rate.

Notably, although inflammatory signaling molecules have received a lot of attention in CRS research, other aspects of their signaling mechanisms (including their receptors) are less well studied.

IL4, *IL22*, and *IL17C* were among the lowest expressed genes, as well as the eosinophilia-related proteins *ECP* (*RNASE3*), *IL6*, and *MPO*. Elevated *IL-4*, and *ECP* (*RNASE3*) has commonly been associated with CRSwNP patients.^{6,18,23,24,29,36,65} The reason for this disparity with studies from elsewhere in the world is unclear, but could reflect differences in the population base. In contrast to European and North American patients, Asian CRSwNP patients tend to be more neutrophilic with elevated *IL-8*, rather than eosinophilic with elevated *IL-4*, *IL-5*, and *IL-13*.^{15,16} Similar distinctions may explain the differences observed in New Zealand patients.

Importantly, the sheer quantity of each marker may not reflect protein activity or roles in the disease process. The focus of this study was to inform efforts aimed at classifying and differentiating among distinct endotypes of CRS. As such, only CRS patients were examined in this study. Inferences cannot be drawn from these data on relative marker expression levels compared to those seen naturally in healthy patients. Lower expression of some markers (such as *IL5*) may constitute clinically meaningful expression compared with healthy mucosa, and these markers may still play essential roles in disease processes in CRS.

Comparisons of sampling methodologies

It was hypothesized that surface swab samples may inadequately reflect tissue transcription patterns. In comparison, cytobrushes are considerably less invasive than mucosal biopsies (although more so than surface swab samples), while cytobrush abrasion of the mucosal surface enables improved sampling of the top tissue layers compared with swab samples. Pairwise comparisons between the 3 sample types highlighted differences in the reported expression levels of some markers based on the sampling methodology used. Reported transcription levels from swab samples for *MUC5AC*, *IL1RN*, *CXCL8* (*IL-8*), *IL33*, *IL5*, *TNF*, *IFNG*, *OSM*, *IL1A*, and *IL17C*, and cytobrush levels for *MUC5AC*, *IL1RN*, *CXCL8* (*IL-8*), *IL33*, *IL5*, and *IL5RA* did not reflect tissue levels. These patterns were similar in overall testing between sample types and in intra-patient comparisons. The presence and activity of some markers may be preferentially localized in the subepithelial tissue, and thus less effectively captured by surface swab or cytobrush sampling. Whether disparities reflect biologically relevant localization patterns warrants further study.

Many of these targets have been reported as markers of CRS in both mucus-based and tissue biopsy-based studies.^{13,18,20,21,27,29,36,60–64,66,67} *IL-5* in particular has repeatedly been identified as an important marker in CRS.^{6,8,13,26} Identifying *IL-5*–high patients in clinic offers the promising possibility of targeted treatment, such as anti-*IL-5* monoclonal antibody therapy.^{5,10,68} The findings here suggest that quantification via noninvasive sampling may not sufficiently represent tissue levels of several important markers for CRS endotyping in the routine clinic setting. However, although the abundances of some markers were underrepresented by non-biopsy sampling here (including *IL33*, *IL5*, and *IL5RA*), all sample types nonetheless identified the presence of each with relative consistency, and these methods may still sufficiently delineate relative transcription levels to a clinically meaningful extent. For example, Tomassen et al.⁸ have distinguished between 3 groups of CRSwNP patients based on *IL-5* protein levels: low or no *IL-5*; moderate *IL-5*; and high *IL-5*. Non-biopsy sampling may sufficiently delineate among these relatively broad brackets, and this warrants further study. In contrast, studies investigating definitive biomarkers of CRS via swab, mucus, or cytobrush sampling may misrepresent

the extent to which some markers are overrepresented (including *MUC5AC*, *IL1A*, *IL1RN*, *CXCL8*, *IL17C*, *IFNG*, *TNF*, and *OSM*) or underrepresented (including *IL33*, *IL5*, and *IL5RA*) in CRS and/or endotypes of CRS.

Once these data were split by CRS phenotype (CRSsNP and CRSwNP), the CRSsNP group appeared to be accounting for the observed differences for *IL33*, *IL5RA*, *FCER1A*, *IFNG*, *IL5*, *OSM*, and *IL17C*. The underlying factors driving the swab and cytobrush discrepancies with tissue patterns in CRSsNP, but not CRSwNP patients, are unclear. Whether marker localization patterns differ in endotypes (and/or by severity) of CRS warrants further study, and will have implications as to the appropriateness of using non-biopsy sampling methods for the characterization of CRS biomarkers and endotypes. Subgrouping by phenotype resulted in low and uneven patient numbers in each group, however. Thus, these results should be interpreted with caution.

Cytobrush samples, and to a lesser extent swab samples, largely tracked the patterns observed in tissue samples for the remaining target genes. In these cases, cytobrush sampling appears to be an acceptable proxy for the assessment of mucosal tissue inflammatory transcription patterns. Swabs were often comparable to cytobrush sampling, and so offer an even less invasive option. However, a greater number of target markers were poorly represented by swab sampling than by cytobrush (including *TNF*, *IFNG*, *OSM*, *IL1A*, and *IL17C*). Additionally, recent studies by Mueller et al.^{51,52} have also identified proteomic assessment of mucus exosome filtrates as a further promising noninvasive (“liquid biopsy”) sampling option. Overall, the findings of this study indicate that these less invasive options acceptably reflect tissue patterns for several putative biomarkers of CRS and could thus be used in clinic and research settings as a proxy for tissue processes.

Limitations and future directions

This study investigated differences between reported transcription patterns of 21 biomarkers of interest for 3 sampling methods in a cohort of 32 patients with CRS. The central focus of this study was the relative efficacy of different sample types to report the transcriptional patterns of biomarkers within CRS patients, with the further aim of differentiating CRS patients by endotypes in mind. As such, non-CRS participants were omitted. Going forward, understanding of how these patterns compare between non-CRS and CRS-affected individuals will be essential in efforts to track putative CRS biomarkers, and will clarify the extent to which non-biopsy-based methods are nonetheless capable of identifying clinically meaningful differences, even in

cases where their reported results do not fully reflect tissue transcription levels.

In the present study, observations of significant differences between the non-biopsy vs biopsy sampling were more prevalent among genes that were also more highly expressed. This study may remain underpowered to detect subtle differences, particularly in the case of genes with lower overall expression. Furthermore, expression levels of several genes were close to or below the limit of detection, including *IL17C*, *IL22*, and *IL4*. Because of the added noise introduced in data close to the limit of detection, results comparing fold changes between sample types for these genes should be interpreted with caution.

Importantly, validation of the efficacy of less invasive sampling methods to represent tissue transcriptional patterns enables the sampling of patients and non-CRS controls outside of the surgical setting. This will vastly increase the prospective recruitment pool and will also better enable the study of dynamics of putative biomarkers and endotypes of CRS within individual subjects over time (due to the less-invasive nature of sampling). Follow-up studies including a larger study cohort, a temporal component, spanning a wide array of the putative CRS endotype spectrum, and including non-CRS control patients for further comparisons, are warranted.

Conclusion

Reported expression via swab and cytobrush sampling for some markers, such as *MUC5AC*, *CXCL8* (IL-8), *IL33*, and *IL5*, differed from patterns observed in matched tissue. Non-biopsy-based studies for these markers may not accurately represent tissue inflammatory processes, and should be interpreted with caution. Further study is required to investigate whether noninvasive sampling nonetheless adequately differentiates between patterns in these markers in a clinically meaningful way. Cytobrush samples, and to a lesser extent swab samples, largely tracked the patterns observed in tissue samples for the remaining target biomarkers. Overall, these data support the use of cytobrush sampling for future studies investigating several important CRS biomarkers, inpatient spatial and temporal patterns, and endotyping in the routine clinic setting to better guide personalized treatment options. 

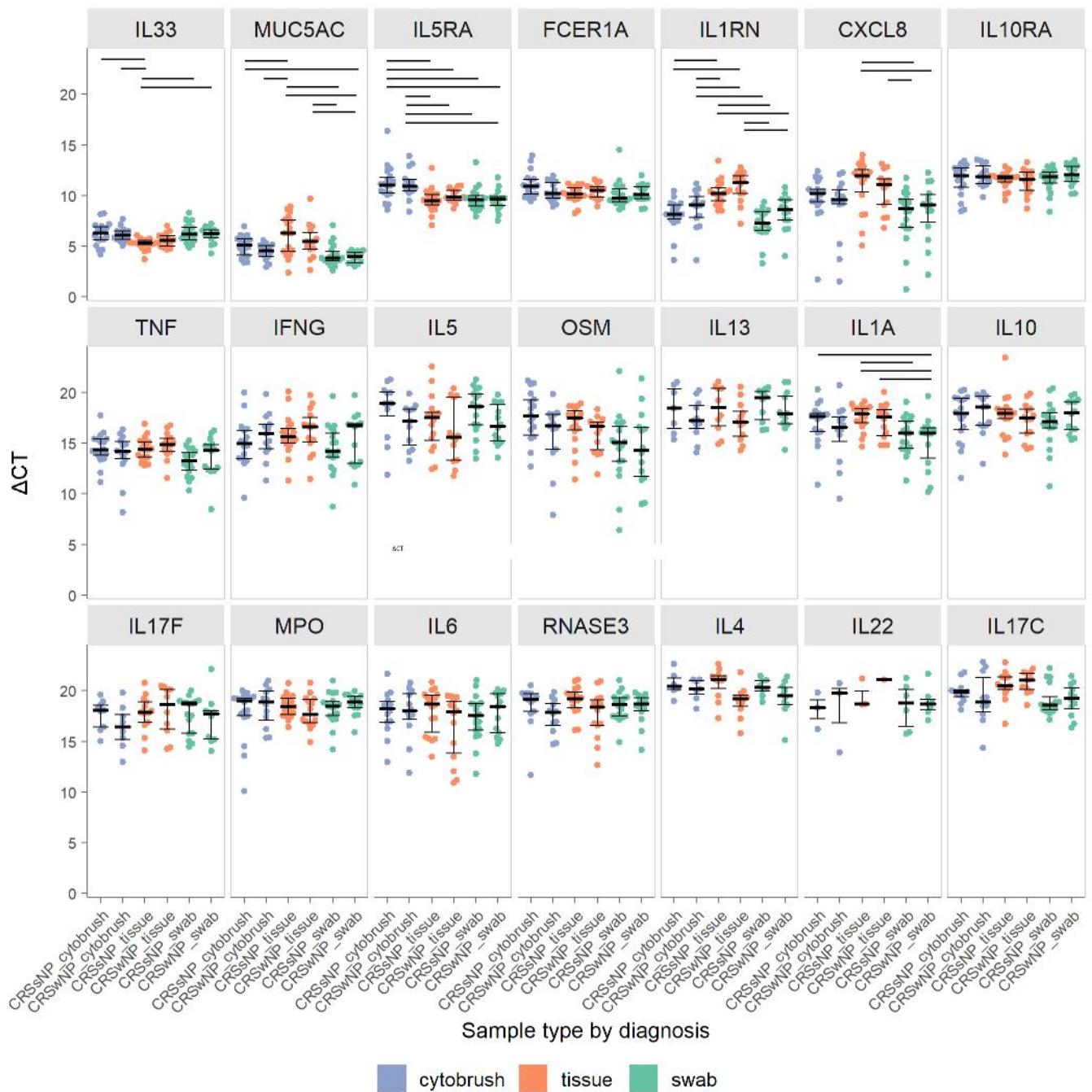
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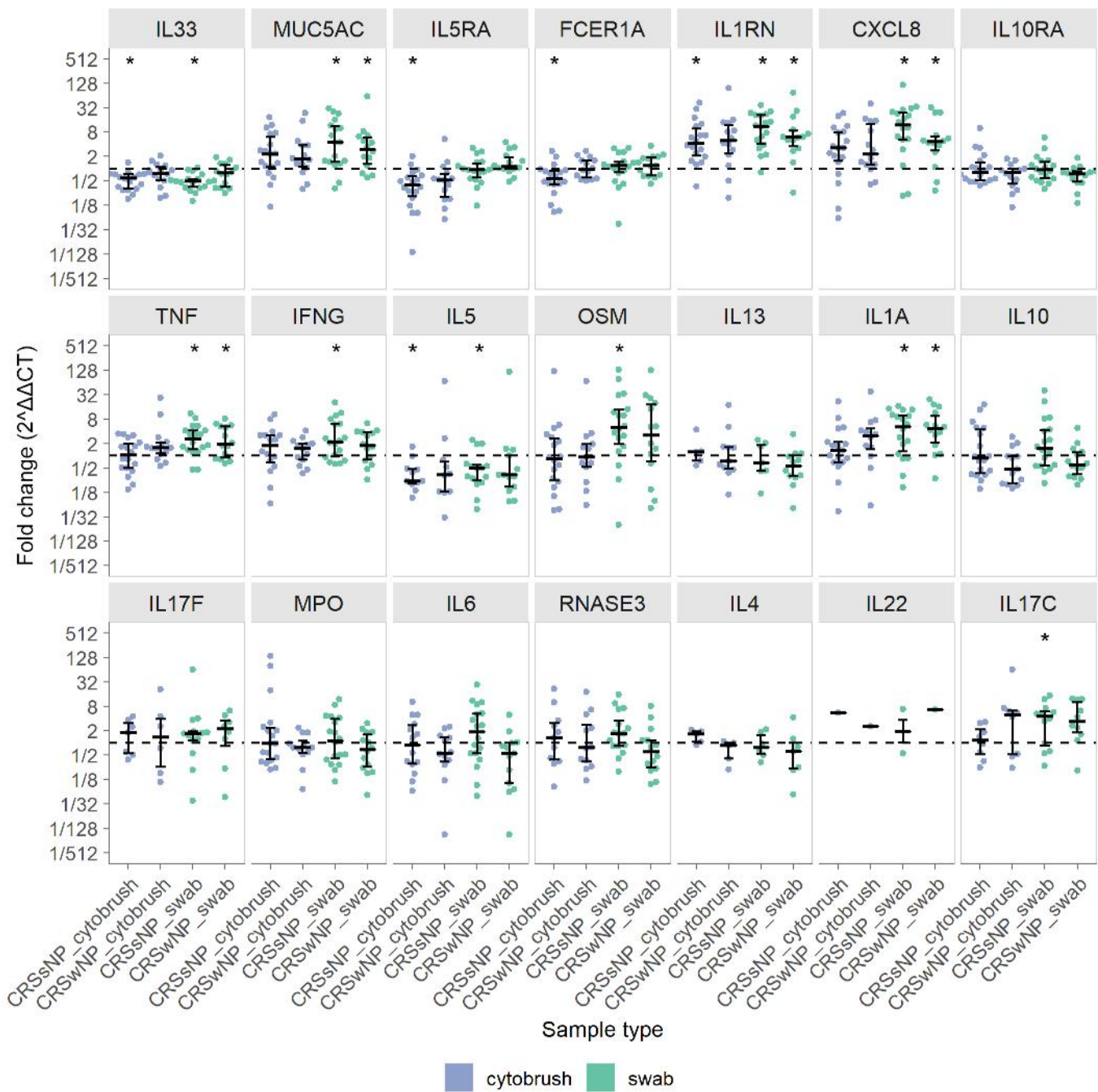
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Supplementary Figure 1. Normalized cycle threshold (delta [Δ]CT) values for each of the 21 target biomarkers in 18 chronic rhinosinusitis without nasal polyps (CRSsNP) patients, and 14 CRS with nasal polyps (CRSwNP) patients. Markers were normalized based on the expression of three genes: *RNA18S*, *GAPDH*, and *HPRT1*. Samples are split by phenotype (CRSsNP and CRSwNP) and the three sample types compared: mucosal cytobrush, tissue biopsy, and swab sampling. Biomarkers are presented in order of highest to lowest expressed genes across tissue samples (lower ΔCT values represent higher reported expression levels). Cross bars represent median and inter-quartile range. Horizontal significance bars (above the plotted points) = significantly different in pairwise comparisons (comparing between the pair joined by the significance bar tips) (FDR adjusted p -value $\alpha = 0.05$).



Supplementary Figure 2. Intra-patient fold differences in biomarker expression reported by each sample type, split by clinical phenotype (18 chronic rhinosinusitis without nasal polyps (CRSsNP) patients, and 14 CRS with nasal polyps (CRSwNP) patients). Log₂ fold differences ($2^{\Delta\Delta CT}$) of cytobrush and swab samples compared with tissue biopsies (with reported expression of tissue biopsies taken as baseline). Cross bars represent median and inter-quartile range. Values greater than 1 represent elevated reported expression, and less than 1 represent reduced reported expression, compared with the tissue sample from the same patient. * = significantly different to reported tissue expression in pairwise comparisons (FDR adjusted p -value $\alpha = 0.05$).

Supplementary Table 1. Normalized cycle threshold (Δ CT) values*

	Cytobrush (n=32)	Tissue (n=32)	Swab (n=32)
IL33	6.1 (4.2 to 8.2)	5.3 (3.7 to 7)	6.2 (4.3 to 8.3)
MUC5AC	4.8 (3 to 7)	5.6 (2.4 to 9.7)	3.9 (2.6 to 7)
IL5RA	11 (8.4 to 16.4)	9.7 (7.1 to 12.7)	9.6 (7.6 to 13.3)
FCER1A	10.7 (8.9 to 14)	10.4 (8.2 to 11.6)	9.9 (8.6 to 14.5)
IL1RN	8.5 (3.6 to 11.2)	10.4 (7.2 to 13.4)	7.8 (3.3 to 10.8)
CXCL8	9.9 (1.5 to 12.4)	11.6 (5.1 to 14)	9 (0.8 to 12.3)
IL10RA	11.9 (8.4 to 13.5)	11.8 (8.7 to 13.3)	11.8 (8.4 to 13.5)
TNF	14.3 (8.2 to 17.7)	14.6 (11.6 to 16.9)	13.6 (8.5 to 16.2)
IFNG	15.2 (9.6 to 20)	15.8 (11.3 to 20.1)	14.9 (8.7 to 19.7)
IL5	18.4 (11.9 to 21.3)	16 (11.8 to 22.6)	17.7 (13.5 to 21.3)
OSM	17.2 (4.5 to 21.2)	16.9 (11.4 to 18.9)	14.9 (6.4 to 22.1)
IL13	17.3 (14.1 to 21)	17.4 (14.3 to 21.1)	18 (14.4 to 21)
IL1A	17.4 (9.5 to 20.7)	17.7 (14.6 to 20)	16 (10.2 to 19.6)
IL10	18 (11.5 to 20.2)	17.7 (13.9 to 23.4)	17.4 (10.7 to 20.4)
IL17F	17.5 (13 to 19.8)	17.9 (14.1 to 20.8)	17.9 (14.1 to 22.1)
MPO	19 (10.1 to 21)	18.3 (15 to 20.8)	18.6 (14.2 to 21)
IL6	18.1 (11.9 to 21.7)	18.4 (10.9 to 20.9)	17.7 (11.8 to 21)
RNASE3	18.6 (11.7 to 20.6)	18.9 (12.7 to 21.1)	18.6 (14.2 to 21)
IL4	20.2 (18.2 to 22.7)	19.6 (15.8 to 22.7)	20.1 (15.1 to 21.4)
IL22	19 (13.9 to 20.7)	19.9 (18.6 to 21.2)	18.7 (15.8 to 21.7)
IL17C	19.6 (14.4 to 22.8)	20.6 (16.8 to 22.8)	18.7 (16.4 to 22.3)

*Normalized cycle threshold values (Δ CT) are given as median (range). Lower cycle threshold values indicate higher cDNA copy numbers in the sample template for the gene of interest. Transcripts are in order of highest to lowest cDNA copy numbers across tissue samples.

Supplementary Table 2. Post-hoc pairwise comparisons of normalized cycle threshold (Δ CT) values*

	P.adj**	CRSsNP_cytobrush - CRSsNP_cytobrush	CRSsNP_cytobrush - CRSsNP_tissue	CRSsNP_cytobrush - CRSsNP_swab	CRSsNP_cytobrush - CRSsNP_swab	CRSsNP_cytobrush - CRSsNP_tissue	CRSsNP_cytobrush - CRSsNP_swab	CRSsNP_cytobrush - CRSsNP_tissue	CRSsNP_cytobrush - CRSsNP_swab	CRSsNP_cytobrush - CRSsNP_tissue	CRSsNP_cytobrush - CRSsNP_swab
IL33	0.006	0.919	0.005	0.149	0.855	0.919	0.012	0.216	0.814		
MUC5AC	0.000	0.306	0.031	0.340	0.071	0.043	0.003	0.065	0.521		
IL5RA	0.001	0.960	0.003	0.022	0.003	0.003	0.003	0.037	0.005		
IL1RN	0.000	0.360	0.000	0.000	0.132	0.666	0.013	0.001	0.017		
CXCL8	0.025	0.501	0.242	0.513	0.131	0.285	0.068	0.200	0.513		
IL1A	0.036	0.303	0.607	0.921	0.163	0.049	0.147	0.303	0.846		
//											
		CRSsNP_cytobrush - CRSsNP_swab	CRSsNP_tissue - CRSsNP_tissue	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab	CRSsNP_tissue - CRSsNP_swab
IL33	0.006	0.919	0.285	0.003	0.012	0.073	0.231	0.787			
MUC5AC	0.000	0.340	0.277	0.000	0.000	0.011	0.007	0.656			
IL5RA	0.001	0.005	0.549	0.889	0.960	0.717	0.574	0.889			
IL1RN	0.000	0.603	0.360	0.000	0.002	0.000	0.000	0.072			
CXCL8	0.025	0.701	0.630	0.011	0.045	0.046	0.121	0.694			
IL1A	0.036	0.418	0.663	0.049	0.021	0.163	0.049	0.488			

* Δ CT values were compared via pairwise *t*-tests, incorporating Benjamini and Hochberg false discovery rate (FDR) calculation. FDR adjusted *p*-values are presented.

** P.adj represents FDR adjusted *p*-values for ANOVA tests against all groups. Variables with significant ANOVA adjusted *p*-values were tested in post-hoc pairwise comparisons.

Supplementary Table 3. Intra-patient gene expression fold differences between sample types

	Cytobrush FDR adjusted <i>p</i> -value*	Cytobrush mean (CI)*	Swab FDR adjusted <i>p</i> -value*	Swab mean (CI)*	Swab vs. cytobrush FDR adjusted <i>p</i> -value*	Swab vs. cytobrush difference CI*
IL33	0.000	0.58 (0.46 to 0.73)	0.000	0.56 (0.45 to 0.7)	0.948	-0.49 to 0.39
MUC5AC	0.007	2.13 (1.36 to 3.33)	0.000	3.54 (2.21 to 5.67)	0.268	-0.18 to 1.66
IL5RA	0.000	0.37 (0.24 to 0.57)	0.671	1.07 (0.82 to 1.4)	0.002	0.81 to 2.27
FCER1A	0.091	0.7 (0.51 to 0.97)	0.710	1.07 (0.78 to 1.45)	0.209	-0.02 to 1.22
IL1RN	0.000	4.53 (2.79 to 7.36)	0.000	7.15 (4.67 to 10.96)	0.324	-0.25 to 1.57
CXCL8	0.004	2.78 (1.6 to 4.85)	0.000	6.07 (3.45 to 10.68)	0.206	0 to 2.24
IL10RA	0.323	0.83 (0.61 to 1.12)	0.200	0.81 (0.62 to 1.05)	0.948	-0.6 to 0.55
TNF	0.292	1.3 (0.89 to 1.9)	0.000	2.31 (1.68 to 3.17)	0.151	0.13 to 1.53
IFNG	0.292	1.33 (0.87 to 2.02)	0.001	2.07 (1.42 to 3.02)	0.268	-0.16 to 1.44
IL5	0.041	0.39 (0.19 to 0.79)	0.021	0.46 (0.26 to 0.82)	0.938	-1.05 to 1.53
OSM	0.799	0.92 (0.47 to 1.8)	0.006	3.65 (1.64 to 8.1)	0.095	0.51 to 3.46
IL13	0.799	1.11 (0.58 to 2.09)	0.076	0.58 (0.35 to 0.97)	0.268	-2.06 to 0.21
IL1A	0.183	1.69 (0.96 to 2.98)	0.000	3.74 (2.33 to 6.01)	0.167	0.1 to 2.19
IL10	0.776	0.88 (0.54 to 1.43)	0.536	1.23 (0.76 to 1.98)	0.536	-0.48 to 1.45
IL17F	0.736	1.31 (0.54 to 3.19)	0.536	1.35 (0.64 to 2.86)	0.948	-1.56 to 1.66
MPO	0.799	1.13 (0.62 to 2.06)	0.641	0.86 (0.54 to 1.38)	0.711	-1.48 to 0.69
IL6	0.292	0.65 (0.34 to 1.23)	0.646	0.82 (0.41 to 1.63)	0.857	-1 to 1.68
RNASE3	0.799	1.15 (0.59 to 2.26)	0.726	1.09 (0.66 to 1.8)	0.948	-1.26 to 1.1
IL4	0.799	0.94 (0.57 to 1.53)	0.295	0.64 (0.32 to 1.27)	0.536	-1.7 to 0.6
IL22	0.292	3.75 (0.03 to 486.03)	0.492	2.92 (0.08 to 106.54)	0.948	-4.87 to 4.15
IL17C	0.292	1.71 (0.76 to 3.82)	0.002	2.97 (1.7 to 5.22)	0.463	-0.57 to 2.17

* Fold differences between cytobrush and swab samples compared with tissue from the same patient were tested via one-sample *t*-test, incorporating Benjamini and Hochberg false discovery rate (FDR). Intra-patient fold differences between cytobrush and swab samples were tested via Welch two-sample *t*-test, incorporating FDR. Significant values are expressed in bold (FDR adjusted *p*-value $\leq$ 0.05).
CI = 95% confidence interval.

Supplementary Table 4. Intra-patient gene expression fold differences between sample type, split by CRS phenotype (CRSsNP, CRSwNP)

	Cytobrush (CRSsNP) FDR adjusted <i>p</i> -value*	Cytobrush (CRSwNP) FDR adjusted <i>p</i> -value*	Swab (CRSsNP) FDR adjusted <i>p</i> -value*	Swab (CRSwNP) FDR adjusted <i>p</i> -value*	Cytobrush (CRSsNP) vs. Cytobrush (CRSwNP) FDR adjusted <i>p</i> -value*	Swab (CRSsNP) vs. Swab (CRSwNP) FDR adjusted <i>p</i> -value*
IL33	0.001	0.181	0.000	0.145	0.494	0.293
MUC5AC	0.075	0.164	0.002	0.025	0.979	0.749
IL5RA	0.007	0.146	0.735	0.180	0.781	0.396
FCER1A	0.042	0.957	0.963	0.376	0.354	0.749
IL1RN	0.001	0.064	0.000	0.006	0.979	0.723
CXCL8	0.085	0.103	0.000	0.014	0.970	0.540
IL10RA	0.958	0.164	0.963	0.060	0.457	0.340
TNF	0.958	0.164	0.002	0.026	0.415	0.824
IFNG	0.726	0.386	0.008	0.145	0.979	0.592
IL5	0.007	0.394	0.019	0.265	0.970	0.824
OSM	0.851	0.957	0.018	0.197	0.970	0.749
IL13	0.803	0.957	0.673	0.114	0.970	0.749
IL1A	0.733	0.208	0.005	0.009	0.765	0.824
IL10	0.803	0.165	0.140	0.197	0.415	0.249
IL17F	0.733	0.957	0.673	0.713	0.979	0.873
MPO	0.726	0.330	0.766	0.180	0.457	0.426
IL6	0.850	0.283	0.501	0.114	0.765	0.249
RNASE3	0.835	0.957	0.146	0.265	0.970	0.249
IL4	0.223	0.294	0.766	0.265	0.415	0.723
IL22	NA	NA	0.766	NA	NA	NA
IL17C	0.958	0.283	0.041	0.060	0.502	0.858

* Fold differences between cytobrush and swab samples compared with tissue from the same patient were tested via one-sample *t*-test, incorporating Benjamini and Hochberg false discovery rate (FDR). Differences between CRSsNP and CRSwNP cytobrush samples, and CRSsNP and CRSwNP swab samples were tested via Welch two-sample *t*-test, incorporating FDR. Significant values are expressed in bold (FDR adjusted *p*-value ≤ 0.05). NA = insufficient data to test.