Proteomic analysis of pediatric sinonasal secretions shows increased MUC5B mucin in CRS

Amarel Saieg[1], Kristy J. Brown[1,2], Maria T. Pena[3], Mary C. Rose[1,2] and Diego Preciado[1,2,3]

BACKGROUND: Chronic rhinosinusitis (CRS) is characterized by mucous overproduction and submucosal gland hyperplasia. The global protein profile of sinonasal secretions in pediatric CRS has not been studied. We hypothesized that MUC5B, a glandular mucin, would be relatively increased in CRS secretions compared to other mucins.

METHODS: Secretions were collected at Children's National Health System (Children's National) from CRS patients undergoing sinus surgery and from control patients without CRS undergoing craniofacial procedures. Proteins were extracted, digested to peptides, and analyzed by mass spectometry. Fold change significance was calculated using the QSpec algorithm. Western blot analysis was performed to validate proteomic findings.

RESULTS: In total, 294 proteins were identified. Although both MUC5B and MUC5AC were identified in a majority of samples, the relative abundance of MUC5B was found to be significantly higher ($P < 0.05$). Western blot data validated these findings. Other proteins with the highest significant positive-fold change in CRS samples were BP1 fold-containing family A member 1, chitinase-3-like protein 1, plastin-2, serpin 10, and BP1 fold-containing family B member 1.

CONCLUSION: Overall, our data demonstrate an increase of MUC5B abundance in the sinus secretions of pediatric patients with CRS.

Chronic rhinosinusitis (CRS) is a common chronic upper respiratory illness that affects more than 16% of the US population (1) and accounts for more than 18 million office visits and direct healthcare costs of over 4 billion dollars a year (2). Although the pathogenesis of CRS has not yet been established at the molecular level, it is well accepted that the pathophysiology reflects a chronic inflammatory process of the paranasal and sinus mucosa, characterized by the overproduction of mucus lasting for 12 wk or more.

Mucin glycoproteins are the primary macromolecular component of mucus. Mucins, as well as other mucosal components, act as physiological barrier against contaminants and bacteria by forming a viscoelastic mucus gel. However, when overproduced, mucins and mucus contribute to the stagnation of secretions, obstruction of sinonasal clearance and chronic infection. To date, 20 mucin glycoproteins have been identified (3). These include the secretory mucins, of which MUC2, MUC5AC, and MUC5B are the best studied, and membrane-tethered mucins, of which MUC1 and MUC4 are presently the best investigated. In healthy lower airways, MUC5AC is typically expressed in the goblet cells, while MUC5B is restricted to the submucosal glands (3). In sinus mucosa, we have shown that MUC5AC is expressed only in goblet cells and that children with CRS do not exhibit goblet cell hyperplasia compared to controls (4) and that MUC5B mucin is expressed in goblet cells, both in control and CRS patients (5). Instead, submucosal glandular hyperplasia is the characteristic phenotype of pediatric CRS and MUC5B is the predominant glandular mucin (5).

To date, there are no studies on MUC5AC and MUC5B levels in sinonasal secretions from children, although MUC5B levels are increased in adult CRS secretions (6). There also appears to be differences in the pathophysiology of CRS in children compared to adults such that results from adult studies may not be applicable to pediatric CRS (7). Several proteomic studies analyzed nasal lavage fluid from adults (8–11) from individuals with and without various forms of sinusitis, but to our knowledge no reports in children. Identifying the exact macromolecular mucin content present in pediatric sinonasal secretions would not only provide clues as to the potential pathophysiology of this condition in children, but could also spark research into innovative ways to specifically target the viscous properties of pediatric sinonasal secretions.

This study was designed to interrogate the global differential expression of proteins, specifically in pediatric CRS secretions, compared to non-CRS secretions using semiquantitative and quantitative techniques in order to determine the correlation between protein abundance and disease state. Given that we have shown that pediatric CRS is histologically characterized by an increase in submucosal glands, a tissue in which MUC5B is well expressed (reviewed in ref. 3), we hypothesized that sinonasal secretions from CRS pediatric patients would contain an overabundance of MUC5B relative to other mucin glycoproteins.

RESULTS

Table 1 lists the demographic information for the patient samples. Mean age of CRS patients was 9.8 y (range 2–14 y). Mean age of control patients was 9.2 y(2–18 y). All CRS subjects had radiographic confirmed evidence of CRS with symptoms lasting greater than 3 mo. Control patients did not have any symptoms of or treatment for CRS and were undergoing surgery for non-CRS indications.
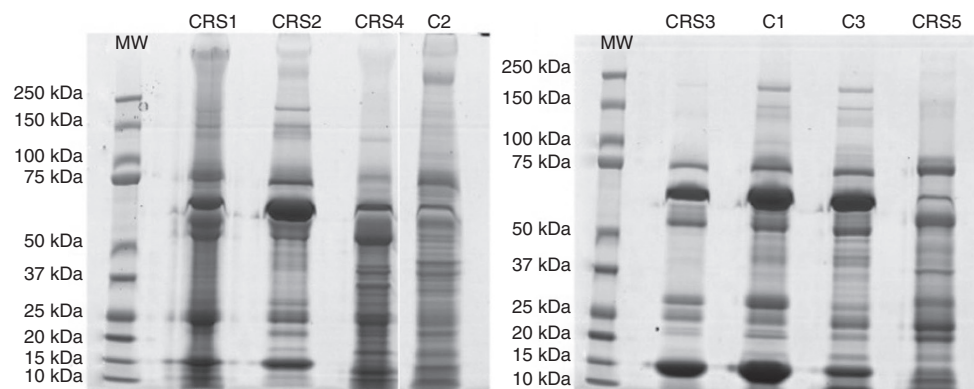
Proteomic Characterization of Secretions

Proteins were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Bio-Safe Coomassie stain (**Figure 1**). For proteomics analysis, the lanes from each sample were serially cut and processed as described in the Methods. A total of 242 proteins were identified by mass spectometry.

Table 1. Chronic rhinosinusitis and control secretions

	Gender	Age (years)	Diagnosis
CRS 1	M	14	CRS
CRS 2	F	16	CRS
CRS 3	F	2	CRS
CRS 4	M	11	CRS
CRS 5	M	4	CRS
CRS 6	F	5	CRS
CRS 7	F	9	CRS
C 1	M	18	Maxillary tooth extraction
C 2	M	16	Midface surgery
C 3	M	14	Midface surgery
C 4	M	3	Sleep apnea
C 5	F	5	Sleep apnea
C 6	M	2	Sleep apnea
C 7	M	15	Midface surgery
C 8	M	2	Sleep apnea

C, control; CRS, chronic rhinosinusitis secretions.

To identify which of the 242 proteins demonstrated the most significant differences in abundance in the two cohorts, the differential expression of each protein was calculated using the QSpec algorithm (12). Proteins were considered to be increased in CRS samples or control samples based on P value ≤ 0.001 and absolute fold change ≥ 1.5. The 47 proteins that meet these criteria are listed in **Table 2** based on the highest fold change in descending order. The protein with the highest significant positive-fold change in CRS samples was bactericidal/permeability-increasing fold containing family A, member 1 (BPIFA1) (log2 fold change = 3.93). The protein with the most significant negative-fold change (decreased abundance in CRS samples) was retinal dehydrogenase 1 (ALDH1A1, log2 fold change = −4.39).

In order to analyze the categories of proteins that were differentially expressed in CRS compared to control sinonasal secretions, the UniProt database was used to analyze the 47 proteins in **Table 2** by Gene Ontology (GO) terms. The top seven most frequent biological processes for CRS and control samples are shown in **Figure 2a,b** respectively. The number of proteins in metabolic and cellular processes were similar for both cohorts. In some processes, e.g., developmental, proteins were more frequently present in control, but not CRS samples. The number of proteins in response to stimulus and biological regulation processes was almost double in the CRS samples. Of note, immune system process proteins were only identified in CRS samples.

Mucin Protein Identification: MUC5B Was Significantly More Abundant Compared to MUC5AC in CRS Samples by Proteomic Analysis

The unique number of peptides and spectral counts for MUC5AC and MUC5B, which are similar in molecular weight and therefore comparable (13), are shown in **Table 3**. MUC5B abundance by spectral count average was significantly higher than MUC5AC spectral count average ($P = 0.043$) in CRS secretions (**Figure 3**). There was no significant difference between the spectral counts for MUC5B compared to MUC5AC in control samples ($P = 0.180$).

Figure 1. Protein content of clinical samples. Coomassie stained 4–12% Bis-Tris gels with desalted sinonasal secretion samples. The first lane of each gel contains molecular weight markers, shown on left (kDa). Each sample lane was loaded with 50 µg total protein. After electrophoresis each lane was serially sliced and in-gel digested for proteomic processing. C, control (nondiseased); CRS, chronic rhinosinusitis secretions.

Table 2. Differentially regulated proteins in CRS and control secretions

Protein name	Common gene name	Fold change
BP1 fold-containing family A member 1	*BP1A*	3.93
Chitinase-3-like protein 1	*CHI3L1*	3.78
Plastin-2	*LCP1*	3.76
Serpin B10	*SERPINB10*	3.72
BP1 fold-containing family B member 1	*BPIFB1*	3.69
Aminopeptidase	*ANPEP*	3.25
Protein S100-A8	*S100A8*	3.19
Serpin B6	*SERPINB6*	3.08
Myeloperxodiase	*MPO*	3.03
Prominin-1	*PROM1*	2.95
Cathepsin G	*CTSG*	2.74
Carcinoembryonic antigen-related cell adhesion molecule 8	*CEACAM8*	2.70
Annexin A6	*ANXA6*	2.56
Galectin-3-binding protein	*LGAL*	2.54
Brain acid soluble protein 1	*BASP1*	2.44
Leukocyte elastase inhibitor	*SERPINB1*	2.33
Mucin-5B	*MUC5B*	2.23
Transketolase	*TKT*	2.2
Myeloblastin	*PRTN3*	2.14
Protein S100-A9	*S100A9*	2.11
Neutrophil gelatinase-associated lipocalin	*LCN2*	1.99
Zymogen granule protein 16 homolog B	*ZG16B*	1.85
Ig α-2 chain C region	*IGHA2*	1.62
Lactotransferrin	*LTF*	1.61
Ig α-1 chain C region	*IGHA1*	1.60
Polymeric immunoglobulin receptor	*PIGR*	1.56
Myosin-9	*MYH9*	1.54
Deleted in malignant brain tumors 1 protein	*DMBT1*	1.52
Peroxiredoxin-6	*PRDX6*	−1.55
Phosphatidylethanolamine-binding protein 1	*PEBP1*	−1.68
Keratin, type II cytoskeletal 8	*KRT8*	−1.69
6-phosphogluconate dehydrogenase, decarboxylating	*PGD*	−1.71
Selenium-binding protein 1	*SELENBP1*	−1.71
Ubiquitin-like modifier-activating enzyme 1	*UBA1*	−1.71
Bisphosphoglycerate mutase	*BPGM*	−1.75
Inter-α-trypsin inhibitor heavy chain H1	*ITIH1*	−1.79
Spectrin α chain, erythrocyte	*SPTA1*	−1.83
Aldehyde dehydrogenase, dimeric NADP-preferring	*ALDH3A1*	−2.16
Inter-α-trypsin inhibitor heavy chain H2	*ITIH2*	−2.22
Apolipoprotein A-IV	*APOA4*	−2.30
Vinculin	*VCL*	−2.39
Alcohol dehydrogenase (NADP+)	*AKR1A1*	−2.64
Keratin, type II cytoskeletal 7	*KRT7*	−2.87
Plectin	*PLEC*	−3.25
Spectrin α chain, brain	*SPTAN1*	−3.49
Retinal dehydrogenase 1	*ALDH1A1*	−4.39

CRS, chronic rhinosinusitis secretions.
Fold change: generated by QSpec algorithm. Proteins with positive fold change are proteins with significant abundance in CRS samples, and proteins with negative fold change are proteins with significant abundance in control samples. Statistical parameters for significant fold change: $P \leq 0.0001$. Fold-change parameter: absolute fold change ≥ 1.5.
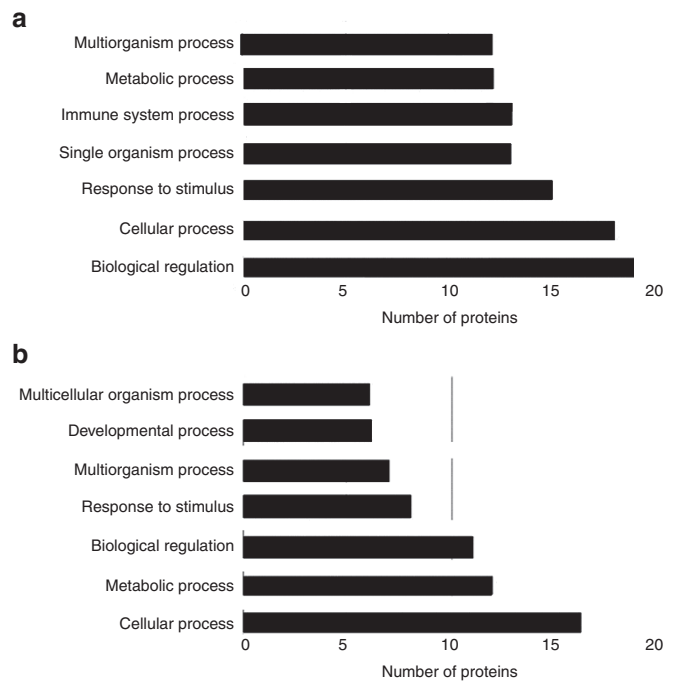
Figure 2. Gene ontology representation of identified proteins in clinical specimens. Significant proteins ($P \leq 0.0001$ and fold change ≥ 1.5) identified in chronic rhinosinusitis secretions (CRS) and control sinonasal secretion samples were compared to the human UniProt Database. The top seven protein enriched Gene Ontology (GO) terms (biological processes) in (**a**) CRS and (**b**) control samples are represented.

Western Blot Analysis of Secretory Mucins in Sinonasal Secretions

The secretory mucins MUC5AC and MUC5B were the only mucin glycoproteins identified in sinonasal secretions by mass spectrometry (MS). Mucins are typically underrepresented in proteomic analysis because of their large size and physical properties (14,15). Thus, we evaluated the abundance of MUC5B and MUC5AC using western blot techniques. **Figure 4a,b** show representative western blots for MUC5B and MUC5AC mucins in sinonasal samples. Due to limited sample availability, only one of the three control samples (sample C2) and four of the five CRS samples (Samples CRS 1–4) that were analyzed by proteomics were available for validation by western blot analysis. In general, there was good correlation between spectral counts and western blot band intensity. The densitometry signal for each protein was normalized to the positive biologic control signal for each blot. The normalized densitometry counts were then averaged per cohort. The average MUC5AC band intensity was lower in CRS than in control secretions, although this did not reach statistical significance ($P = 0.06$). On the other hand, parallel to the proteomics data, there was an increase (7.5-fold) in MUC5B intensity in CRS relative to control secretions, although this was not statistically significant ($P = 0.11$) (**Figure 4c**).

DISCUSSION

For this study, we aimed to gain a better understanding of the protein components in sinonasal secretions from CRS patients

Table 3. Unique peptides and spectral counts identified in samples analyzed by proteomics

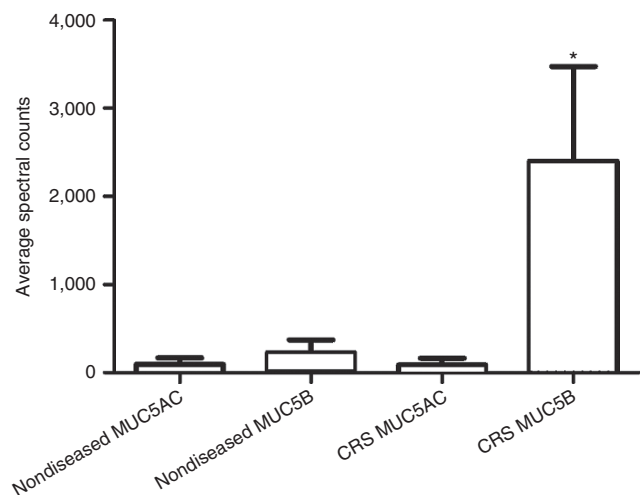| Sample	MUC5B		MUC5AC	
	Unique peptides	Spectral count	Unique peptides	Spectral count
CRS 1	29	6,419	10	394
CRS 2	15	1,626	2	44
CRS 3	1	13	0	0
CRS 4	19	2,316	14	0
CRS 5	3	1,626	0	0
C 1	0	0	0	0
C 2	25	496	24	241
C 3	11	182	5	39

C, control; CRS, chronic rhinosinusitis secretions.

Figure 3. Spectral count of MUC5AC and MUC5B. Average normalized spectral count of MUC5B peptides compared to MUC5AC peptides in sinonasal secretions from pediatric chronic rhinosinusitis secretions (CRS) and control patients. Significantly more MUC5B was detected compared to MUC5AC in CRS samples (*P = 0.043, compared to MUC5AC in CRS), but not in control samples. White bar: nondiseased MUC5AC; black bar: nondiseased MUC5AB; gray bar: CRS MUC5AC; hatched bar: CRS MUC5B.

compared to sinonasal secretions from control patents. In contrast to other studies, we utilized sinonasal secretions obtained during surgery from CRS and control patients directly from the osteomeatal region, rather than nasal lavage fluid. Utilizing label-free proteomic techniques, we successfully created a protein profile showing the varying levels of expression of 294 proteins in sinonasal secretions from control and CRS pediatric patients. A large number of the proteins identified as having significant fold change in CRS sinonasal secretions were involved in innate immune response.

Most of the proteins with significant fold changes identified in our CRS sinonasal samples were assigned to 'immune system processes' by GO, supporting the concept that sinonasal mucosa in CRS patients is in a chronic inflammatory state. Several of the innate immunity proteins that show increased expression in CRS secretions in our study, e.g., BPIFA1, DMBT1, S100A8, S100A9, LTF, MYH9, and IGHA1, have been reported as being dysregulated in studies of CRS nasal lavage fluid or sinus tissues (6,11,16–19). These proteins are discussed below.

BPIFA1, which has the highest fold change in CRS sinonasal secretions, is a member of the BPI or PLUNC family of proteins (20) found in secretions from the upper airways, nose, and mouth. While the functions of BPI proteins have not yet been fully elucidated, they appear to have multifunctional roles in airway immune defense. Other proteomic studies identified high expression of BPIFA1 in CRS nasal mucosa tissue and verified this observation by immunohistochemistry (16). Since BPIFA1 is well expressed in submucosal glands in the respiratory tract (21,22), its overexpression in CRS sinonasal secretions further supports submucosal gland hyperplasia in CRS sinus mucosa.

DMBT1, also known as GP340, is a glycoprotein implicated in immune response (23), mucosal inflammation and epithelial regeneration (24). Its scavenger receptor cysteine rich domains (25) and glycan side chains (26) facilitate its binding to various pathogens (25), allowing it to function as part of the innate defense mucosal mechanism to remove bacteria, viruses or other infectious agents. Interestingly, DMBT1 is strongly associated with MUC5B in respiratory tract secretions (27).

The S100 proteins, specifically S100A8 and S100A9, are proinflammatory mediators that induce chemotaxis of neutrophils and monocytes. Each has been identified in CRS samples. S100A9 is overexpressed in nasal secretory cells from CRS patients with nasal polyps (28), as well as nasal mucus (29) and nasal lavage fluid from CRS patients (19). S100A8 is overexpressed in sinonasal mucosa tissue from patients with CRS with nasal polyps (30,31).

LTF, MYH9, and IGHA1 are also increased in CRS samples. Proteomic studies have shown overexpression of LTF, an antimicrobial protein, in the nasal lavage fluid of patients with acute sinusitis (19), asthmatic CRS (11), smokers (10), and seasonal allergic rhinitis patients (32), as well as nasal mucus and tissue from CRS patients (29,33). MYH9, which aids in cell motility and maintains cell shape (34), and IGHA1, which acts in immune system defense (35), also exhibit increased levels in proteomic studies on nasal lavage fluid from acute sinusitis (8) and asthmatic CRS (11) patients.

Mucins in Sinonasal Secretions

Mucin data from our proteomic and western blot analysis indicated a trend of overexpression of MUC5B mucin in pediatric CRS sinonasal secretions relative to MUC5AC. This result is not unexpected, based on studies showing an increase in submucosal glands in CRS patients (5,36) and expression of MUC5B in the mucosal cells in submucosal glands of the sinus mucosa (5). Proteomic analysis showed that MUC5B was significantly overexpressed in CRS samples, whereas MUC5AC did not reach statistical significance. In line with our results, MUC5B, but not MUC5AC, has been identified by proteomic analyses of nasal mucus from CRS patients with nasal polyps (29).

Viswanathan *et al.* evaluated MUC5B and MUC5AC mucin expression in sinus mucosa samples by ELISA to show a
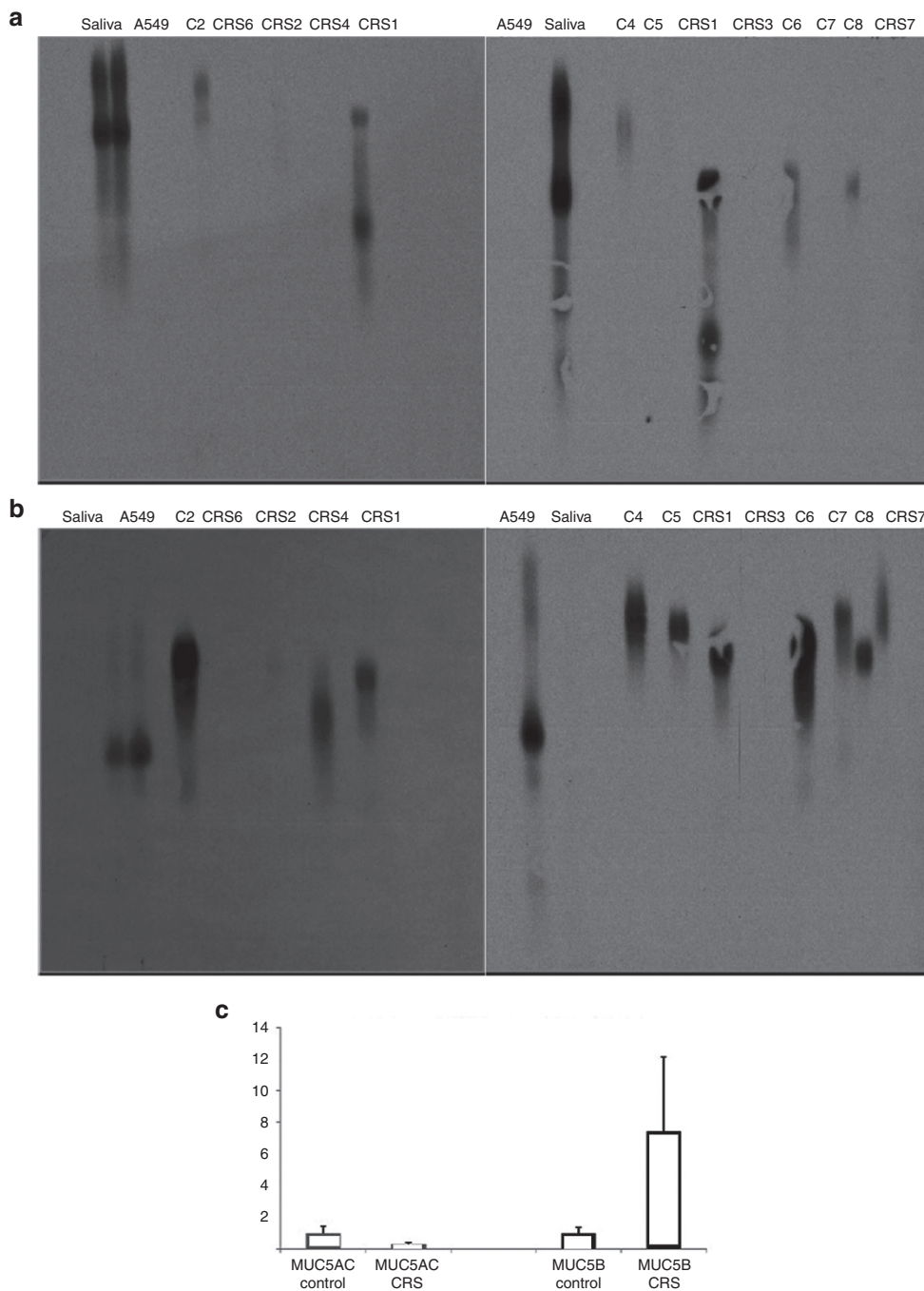
Figure 4. Western blots of mucin glycoproteins. (**a**) Representative western blot of MUC5AC mucin in sinonasal secretions from children with or without chronic rhinosinusitis secretions (CRS). Samples were electrophoresed on 1% agarose gels and immunostained at a dilution of 1:4,000 using an anti-MUC5AC rabbit polyclonal antibody (see Methods). 30 µg protein from sinonasal secretions was loaded. The sample numbers are listed above the lane. C, control (nondiseased). Saliva (30 µg) was used as a negative internal control and A549 cell lysates (30 µg) as a positive internal control for MUC5AC mucin. (**b**) Representative western blot analysis of MUC5B in sinonasal secretions from children with or without CRS. The membrane used in **a** was stripped and immunostained with an anti-MUC5B rabbit polyclonal antibody (see Methods) at a dilution of 1:300. 30 µg of protein for all samples was loaded. This time, A549 cell lysates were used as a negative internal control and saliva as a positive internal control for MUC5B mucin. (**c**) The average fold change of MUC5AC band intensity decreased in disease to a level 0.298 of control band intensity, although this did not reach statistical significance ($P = 0.058$). On the other hand, parallel to the proteomics data, there was a marked 7.47-fold change increase in MUC5B signal in disease relative to control, although this did not reach statistical significance either ($P = 0.11$).

statistically significant increase in MUC5B levels in CRS compared to controls with no difference in MUC5AC levels between the cohorts (6); a finding that is also consistent with our proteomic data.

Conclusions

To our knowledge, this study represents the first global protein profile analysis of pediatric sinonasal secretions from patients with and without CRS, characterizing the presence of proteins

in CRS samples which are to likely to play a role in the inflammatory nature of CRS. Furthermore, MUC5B has an increased abundance trend compared to MUC5AC in CRS patients, likely reflecting the increased glandular hyperplasia characteristic of the disease process. Many of the significantly increased proteins in CRS sinonasal secretions are implicated in airway inflammatory reaction and immune response and some might be useful to further investigate as potential biomarkers for CRS.

METHODS

Sample Collection and Preparation
Sinonasal secretions were collected from pediatric patients according to the institutional guidelines at Children's National Medical Center (Children's National). Studies were approved by the Institutional Review Board of Children's National. All subjects/families signed informed consent for participation in this study. Control secretions were obtained from patients without known CRS disease undergoing craniofacial and/or neurosurgical surgeries. CRS secretions were collected from patients undergoing functional endoscopic sinus surgery for CRS. Exclusion criteria for CRS patients included: Patients with CRS who do not require surgery. Exclusion criteria for controls included: a history of sinonasal surgery, current sinonasal infection, sinonasal or allergic symptoms within the previous 3 mo, and/or treatment with topical nasal steroids within 1 mo or antihistamines within 3 mo before surgery. Computed tomography and or magnetic resonance imaging for controls all had normal paranasal sinuses, these images had all been obtained for clinically indicated reasons and never for sole purposes of this study. Using endoscopic guidance, samples were collected directly from the osteomeatal complex, put on ice, and transferred to the lab. Endoscopically directed sampling of secretions from the osteomeatal complex have been shown to be an accurate representation for the properties of secretions from inside the sinuses, at least in terms of bacterial presence (37,38). Secretions from the left and right osteomeatal complexes were combined and centrifuged at 1,100 rpm at 4 °C for 5 min. The supernatant was transferred to a clean tube and 1 ml of ice cold phosphate-buffered saline was added. The same supernatant was centrifuged at 1,500 rpm for 10 min at 4 °C. Samples were then desalted and concentrated on an Amicon Ultra-4 3K filter units (Millipore, Billerica, MA). Protein content was analyzed by a bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL). Eight secretion samples were used for proteomic analysis (three control, five CRS). Four additional samples (three control and one CRS), were used for Western analyses.

Proteomic Sample Preparation
A 100 μg of total protein for samples were resuspended (NuPAGE LDS with reducing agent) and subjected to one-dimensional SDS gel electrophoresis using a 4–12% Bis-Tris polyacrylamide gel (Life Technologies, Carlsbad, CA) at 200 V for 50 min. The gel was fixed with a 50% methanol, 45% ddh$_2$O, and 5% acetic acid solution and stained with Bio-Safe Coomassie for protein visualization. Each gel lane was serially sliced into 33 segments and in-gel digested with trypsin as previously (39).

MS and Protein Identification
MS analysis and data processing was carried out using methods as previously reported by our group (39). Briefly, tryptic peptides were injected via an autosampler and loaded onto a C18 trap column followed by separation on a C18 reverse-phase column using an Eksigent nano-HPLC system (Dublin, CA). The Linear Ion Trap Mass Spectrometer (LTQ) (ThermoFisher Scientific, Waltham, MA) was operated in data-dependent mode with dynamic exclusion in which one cycle of experiments consisted of a full-MS (300–2,000 m/z) survey scan and five subsequent MS/MS scans of the most intense peaks. Each survey scan file was searched for protein identification using the Sequest algorithm in the Bioworks Browser software (ThermoFisher Scientific, version 3.3.1) against the Uniprot database indexed for human, fully tryptic peptides, two missed cleavages, and potential

modification of oxidized methionine (15.9949 Da). Data generation parameters were Peptide Tolerance of 1.5 Da and Fragment Ion Tolerance of 1 Da. Search results were uploaded into ProteoIQ software (version 2.3.07, Premier Biosoft, Palo Alto, CA) and filtered based on the following criteria: XCorr > 1.9, peptides > 6 amino acids, 2 spectra per peptide, 0.98 peptide probability and 0.95 protein probability. Proteomics data were filtered using QSpec's statistical algorithm to determine which proteins had significant fold-change values. Data were submitted through the QSpec web submission page (12) (Ann Arbor, MI). The QSpec algorithm, is tailored for the analysis of differential proteins expression using label-free spectra count data and small sample numbers (12). To account for blood and other contamination, data were normalized to total scan counts after subtraction of albumin, hemoglobin, and keratins. This was done in order to remove the likely confounding contribution of these overabundant proteins to the overall proteinacious content of the biospecimen. Dealing with blood contamination in proteomic analyses of biospecimens remains a standard problem.

Western Blotting
Western blot analysis was performed using established protocols in our laboratories (39,40). For mucin protein blotting, samples containing 40 μg total protein were separated by electrophoresis in 1.0% agarose gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The positive control for MUC5B was human saliva to which protease inhibitor cocktail (Sigma, St Louis, MO) was added at a 1:100 dilution on collection and for MUC5AC was A549 lung carcinoma cell lysates. For MUC5AC immunostaining, the membrane was probed using LUM5-1 (41), a rabbit polyclonal anti-MUC5AC antibody generously provided to us by Dr Mehmet Kesimer, University of North Carolina at Chapel Hill at a 1:4,000 dilution in a 2.5% milk solution in phosphate-buffered saline for 1 h at room temperature followed by incubation at 4 °C overnight. The antibody (LUM5-1 epitope) was raised against the sequence RNQDQQGPFKMC in the cysteine rich nontandem repeat domains of MUC5AC. MUC5B expression was detected with a rabbit polyclonal α-MUC5B H-300 (Santa Cruz Biotechnology, Dallas, TX) at 1:300 dilution. The respective intensities of positive bands relative to the positive control were densitometrically semiquantified with Quantity One 4.3.1 image processing software (BioRad, Hercules, CA) using equal sized box-shaped markers.

Statistical Analysis
Protein fold change was considered to be significant if *P* value (or false discovery rate) was less than or equal to 0.0001. Additionally, only proteins with an absolute fold change greater than or equal to 1.5 were taken into account. Mann–Whitney tests for nonparametric data were used for densitometry analyses of western blots.

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