

Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis

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Background: Nasal mucus is the first-line defense barrier against (aero-) allergens. However, its proteome and function have not been clearly investigated.

Objective: The role of nasal mucus in the pathophysiology of allergic rhinitis was investigated by analyzing its proteome in patients with allergic rhinitis (n = 29) and healthy control subjects (n = 29).

Methods: Nasal mucus was collected with a suction device, tryptically digested, and analyzed by using liquid chromatography–tandem mass spectrometry. Proteins were identified by searching the SwissProt database and annotated by collecting gene ontology data from databases and existing literature. Gene enrichment analysis was performed by using Cytoscape/BINGO software tools. Proteins were quantified with spectral counting, and selected proteins were confirmed by means of Western blotting.

Results: In total, 267 proteins were identified, with 20 (7.5%) found exclusively in patients with allergic rhinitis and 25 (9.5%) found exclusively in healthy control subjects. Five proteins were found to be significantly upregulated in patients with allergic rhinitis (apolipoprotein A-2 [APOA2], 9.7-fold; α 2-macroglobulin [A2M], 4.5-fold; apolipoprotein A-1 [APOA1], 3.2-fold; α 1-antitrypsin [SERPINA1], 2.5-fold; and complement C3 [C3], 2.3-fold) and 5 were found to be downregulated (antileukoproteinase [SLPI], 0.6-fold; WAP 4-disulfide core domain protein [WFDC2], 0.5-fold; haptoglobin [HP], 0.7-fold; IgJ chain [IGJ], 0.7-fold; and Ig hc V-III region BRO, 0.8-fold) compared with levels seen in healthy control subjects.

Conclusion: The allergic rhinitis mucus proteome shows an enhanced immune response in which apolipoproteins might play an important role. Furthermore, an imbalance between cysteine proteases and antiproteases could be seen, which negatively affects epithelial integrity on exposure to pollen protease activity. This reflects the important role of mucus as the first-line defense barrier against allergens. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Nasal mucus, proteome, proteomics, allergic rhinitis, mass spectrometry

Mucus is the first-line defense barrier of the upper respiratory tract, and its proper production and transport maintain a healthy and patent airway and protect the epithelium.¹⁻⁴ Mucus mainly consists of polypeptides, cells, and cellular debris,^{2,3} but little is known about the distinct proteins that comprise the nasal mucus proteome. Casado et al⁵ were the first to publish a proteomics study about nasal mucus in healthy control subjects, identifying 111 different proteins.

According to the Allergic Rhinitis and its Impact on Asthma guidelines, allergic rhinitis is a disorder of the nose induced after allergen exposure by IgE-mediated inflammation of the nasal mucosa, during which rhinorrhea is one of the cardinal symptoms.⁶ Thus nasal mucus must somehow be involved in its pathophysiology. As a highly prevalent disease, especially in Western countries, allergic rhinitis is a huge problem for patients and the health care system.⁶ The aim of our study was to investigate the nasal mucus proteome in patients with allergic rhinitis compared with healthy control subjects. As a part of this first-line defense barrier against harmful agents, nasal mucus proteins are most likely involved in physiologic and pathologic processes. The major question we asked was whether proteomic changes are involved in allergic rhinitis or whether there are no differences compared with healthy control subjects. On the one hand, we sought to shed light on how immune system responses are represented through the mucus proteome in patients with allergic rhinitis. On the other hand, we hypothesized that the mucus proteome might confirm and extend theories of transepithelial transport of allergens.⁷ Regarding the latter point, pollen grains were found to contain proteinases,⁸ which can degrade tight junctions,⁹ leading to allergen penetration through the epithelial barrier. Thus we were also interested in the presence of innate protease inhibitors and proteases and whether their balance was altered in the mucus of patients with allergic rhinitis compared with that of healthy control subjects.

The objective of this proteomic study was to obtain a large spectrum of proteins present in nasal mucus and identify key proteins, such as apolipoproteins and cysteine protease inhibitors. The function of these proteins could reflect their involvement in immune responses leading to allergic rhinitis, whereas other proteins could reduce the immune response and deactivate harmful pollen content, such as proteases acting as defense mechanisms in healthy control subjects. Targeting these proteins for interventions might offer new therapeutic strategies on the mucus level because it is the first-line defense barrier of the nasal mucosa.

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Abbreviations used

A2M:	α 2-Macroglobulin
APOA1:	Apolipoprotein A-1
APOA2:	Apolipoprotein A-2
APOA4:	Apolipoprotein A-4
APOB:	Apolipoprotein B-100
BPIFA1:	BPI fold-containing family A member 1
BPIFB1:	BPI fold-containing family B member 1
C3:	Complement C3
C5:	Complement C5
GRN:	Granulin
HP:	Haptoglobin
IGJ:	IgJ chain
LC-MS/MS:	Liquid chromatography–tandem mass spectrometry
LTF:	Lactotransferrin
LYZ:	Lysozyme C
RNASE2:	Nonsecretory ribonuclease
SC:	Spectral count
SERPINA1:	α 1-Antitrypsin
SLPI:	Antileukoproteinase
VTN:	Vitronectin
WFDC2:	WAP 4-disulfide core domain protein

METHODS**Patients**

Fifty-eight subjects (31 male and 27 female subjects) were included in this study. The mean age was 34 years (range, 20–58 years), and there were 29 (50%) patients with allergic rhinitis and 29 (50%) healthy control subjects. Allergy status was verified by using skin prick tests (Allergopharma GmbH & Co KG, Reinbek, Germany) and specific IgE measurement (ImmunoCAP; Thermo Fisher Scientific, Vienna, Austria), respectively (see [Table E1](#) in this article's Online Repository at www.jacionline.org). Patients sensitized to house dust mite or animals only were excluded to avoid bias because of the small sample size. Thus only patients with symptoms during the pollen season were considered for evaluation. Patients with acute sinusitis, chronic sinusitis, or both, as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines,¹⁰ were excluded from the study. Furthermore, patients with malignant tumors or any other infectious or cardiopulmonary disease or those treated with systemic or topical drugs, such as antihistamines, corticosteroids, antibiotics, antifungal agents, or any other immunomodulatory drugs, in the 4 weeks before the study were excluded. Informed consent was obtained from all subjects (both patients with allergic rhinitis and healthy control subjects) before entering the study. The study was approved by the Institutional Review Board of the Medical University Graz.

Sample collection

A special suction device (Sinus Secretion Collector; Medtronic Xomed, Jacksonville, Fla) was used to collect nasal mucus. Without previous interventions (decongestants and local anesthetics), untreated mucus was obtained under endoscopic control from the nasal cavity and middle meatus, with meticulous care taken not to touch the mucosa. Then mucus was deep frozen at -80°C before processing for liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Proteomic analysis

Sample preparation, LC-MS/MS analysis, and LC-MS/MS data analysis are described in detail in the [Methods](#) section in this article's Online Repository at www.jacionline.org and reported according to “minimum information about a proteomic experiment.”¹¹ In brief, equal amounts of protein digests were separated by means of nano-HPLC and measured online by using tandem mass spectrometry. Spectra were matched to the SwissProt human protein database with appropriate software (Spectrum Mill, Proteome Discoverer,

and Mascot). Spectral counting of the total peptides identified (ie, number of MS/MS spectra matched to a protein) was used to compare the relative protein abundances of the same protein between groups.^{12,13} Identified proteins were annotated by using data from UniProt (www.uniprot.org), the PANTHER classification system (www.pantherdb.org), and DAVID (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md; <http://david.abcc.ncifcrf.gov/>). Enrichment analysis was performed with BINGO 2.44¹⁴ in Cytoscape 2.8.1 software (www.cytoscape.org).¹⁴

Statistical analysis

For statistical analysis of group differences of spectral counting data, only proteins with a mean spectral count (SC) of 4 or greater in either group were accepted.¹² SC data are presented as means with SDs or SEMs. The Mann-Whitney *U* test was used to identify significantly altered proteins between groups with SPSS 18.0 software (SPSS, Chicago, Ill). A *P* value of less than .05 was considered significant.

For statistical analysis of enrichment data created with BINGO/Cytoscape, hypergeometric tests were performed and corrected with Benjamini & Hochberg false discovery rate correction at a significance level of .05.

Western blot analysis

Twenty micrograms of lysed protein from different nasal mucus samples was separated by 4% to 20% denaturing reducing SDS-PAGE (Bio-Rad Laboratories, Vienna, Austria). Separated proteins were transferred onto nitrocellulose membranes by means of semidry blotting for 1 hour at 180 mA. Total transferred protein was detected with Pierce MemCode (Thermo Fisher Scientific) reversible stain and imaged on a ChemDocXRS (Bio-Rad Laboratories). Membranes were blocked in blocking buffer (5% skim milk in Tris-buffered saline–Tween buffer [20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween 20, pH 6.7]) for 1 hour at room temperature and then incubated with primary antibodies overnight at 4°C . Primary antibodies specific to α 1-antitrypsin (SERPINA1; product no. 9400; Abcam, Cambridge, United Kingdom), α 2-macroglobulin (A2M; product no. 58703; Abcam), haptoglobin (product no. 13429; Abcam), complement C3 (C3; product no. 97462; Abcam), and apolipoprotein A-2 (APOA2; product no. 24241; Abcam) were used for protein detection. After washing with Tris-buffered saline–Tween, the membrane was incubated with secondary antibodies (goat anti-mouse or anti-rabbit IgG–horseradish peroxidase [HRP] conjugates, Abcam) for 1 hour at room temperature. Immunocomplexes were visualized with Pierce ECL chemiluminescent substrate (Thermo Fisher Scientific). Densitometric evaluation was performed with Image Lab 4.1 software (Bio-Rad Laboratories). Volumes of protein bands were determined by using global background subtraction and normalized on total protein detected in the lane by using MemCode. Means and SEMs of 5 patients in each group were calculated.

RESULTS

Using a shotgun proteomics approach, we identified the nasal mucus proteomes of 29 patients with allergic rhinitis and 29 healthy control subjects (see the [Methods](#) section and [Table E1](#) in this article's Online Repository for patients' characteristics and method details). The mean mucus protein concentration was 3.34 mg/mL (SD, 4.1 mg/mL) in patients with allergic rhinitis and 2.88 mg/mL (SD, 3.7 mg/mL) in healthy control subjects, which did not reach significance ($P = .57$). In shotgun proteomics the total sample is digested by a protease, typically trypsin, and the resultant peptides are separated by means of liquid chromatography and sequenced by means of tandem mass spectrometry. Proteins are identified by matching experimental and theoretic peptide spectra and statistically validated. The number of identified unique (ie, specific) peptides for a protein and the protein

sequence coverage reflect the reliability of the identification. By using this approach, 267 proteins were identified in nasal mucus over all 29 patients with allergic rhinitis and 29 healthy control subjects (see [Table E2](#) in this article's Online Repository at www.jacionline.org).

To compare the relative levels of individual proteins between the 2 groups, we applied spectral counting, which is an identity-based label-free quantitation method and therefore well suited to analyze large sample numbers.^{12,13} The SC is the total number of detected peptide spectra matched to each protein. A mean SC of 1165 (SD, 201) was measured in mucus of patients with allergic rhinitis ($n = 29$) and 1058 (SD, 300) in mucus of healthy control subjects ($n = 29$), respectively, when injecting the same amount of digested protein (2 μ g) into the mass spectrometer.

The number of detected peptides depends on (1) the number of possible peptides and thus on individual protein size and sequence, as well as hydrophobicity, and on the used analytic set-up and (2) on ionization properties and thus on the peptide sequence and sample matrix. Accordingly, although relative comparison of individual protein amounts between samples is reliably performed in highly similar samples by using spectral counting, absolute quantitation of individual proteins within 1 sample would require alternative methods using internal standards. However, this is beyond the scope of this study, in which we aimed at identifying relative differences in mucus proteome abundance between patients with allergic rhinitis and healthy control subjects.

The control nasal mucus proteome comprises 247 proteins, which were identified with at least 2 unique peptides over all 29 healthy control subjects (see [Table E3](#) in this article's Online Repository at www.jacionline.org). The most abundant proteins with a mean SC of greater than 20 were albumin (ALB), lactotransferrin (LTF), Ig alpha-1 chain C region, the polymeric immunoglobulin receptor, prolactin-inducible protein, Ig kappa chain C region, BPI fold-containing family B member 1 (BPIFB1), lysozyme (LYZ) C, and Ig lambda-2 chain C region. The found ALB originates from plasma. IgA consisting of the heavy α chain and the light κ and/or λ chains is secreted locally into the nasal mucosa by means of transport across the endothelial cells through binding to the polymeric immunoglobulin receptor. The BPI fold-containing family B member is also known as long palate, lung, and nasal epithelium carcinoma-associated protein 1 and originates from the nasal epithelium. Although prolactin-inducible protein is produced by salivary glands, LTF and LYZ are produced by submucosal glands, respectively.

Next we compared the control nasal mucus proteome with the allergic rhinitis proteome. In the present study 222 (83%) of the 267 identified proteins were present in both patients with allergic rhinitis and healthy control subjects, whereas 20 (7.5%) of the 267 proteins were found exclusively in patients with allergic rhinitis and 25 (9.5%) of the 267 proteins were found exclusively in healthy control subjects (see [Table E2](#)). In these proteins the following 6 biological processes were significantly enriched in patients with allergic rhinitis and none in healthy control subjects compared with the total human proteome: response to stress, response to external stimulus, behavior, multicellular organism development, cellular component organization, and regulation of the biological process.

The 51 most abundant proteins with a mean SC of 4 or greater in either group are depicted in [Fig 1](#) and compared between patients with allergic rhinitis and control subjects by means of

spectral counting. Of the most abundant proteins, ALB, IgA (heavy and light chains), and BPIFB1 levels were slightly increased in patients with allergic rhinitis, whereas LTF, LYZ, and polymeric immunoglobulin receptor levels were slightly decreased, but none of these changes were statistically significant.

Significantly more abundant proteins and enriched biological processes in patients with allergic rhinitis

With a mean SC of 4 or greater, the following 5 proteins were significantly more abundant in patients with allergic rhinitis: APOA2, 9.7-fold; A2M, 4.5-fold; apolipoprotein A-1 (APOA1), 3.2-fold; SERPINA1, 2.5-fold; and C3, 2.3-fold ([Table I](#) and [Fig 1](#)).

Enrichment analysis obtained by using BINGO software (GOSlim_generic) revealed 12 enriched biological processes in patients with allergic rhinitis compared with the total human proteome, 8 of which were also increased in healthy control subjects. The remaining 4 were exclusively found in patients with allergic rhinitis: lipid metabolic process, transport, symbiosis encompassing mutualism through parasitism, and response to external stimulus ([Fig 2, A](#), and [Table II](#)).

Significantly less abundant proteins and depleted biological processes in patients with allergic rhinitis

Considering a mean SC of 4 or greater, levels of the following 5 proteins were significantly decreased in patients with allergic rhinitis compared with a higher abundance in healthy control subjects: antileukoprotease (SLPI), 0.6-fold; WAP 4-disulfide core domain protein (WFDC2), 0.5-fold; haptoglobin (HP), 0.7-fold; IgJ chain (IGJ), 0.7-fold; and the Ig hc V-III region BRO, 0.8-fold. Six biological processes were significantly reduced and not present in patients with allergic rhinitis compared with healthy control subjects: carbohydrate metabolic processes, generation of precursor metabolites and energy, organelle organization, cytoskeleton organization, cell differentiation, and cellular component organization ([Fig 2, B](#), and [Table III](#)).

Five significantly altered proteins for which commercial antibodies were available were additionally assessed by means of Western blotting in the nasal mucosa of 5 patients with allergic rhinitis and 5 healthy control subjects, respectively. The detected bands for APOA2 (12 kDa), A2M (160 kDa), SERPINA1 (55 kDa), and C3 (110 kDa) were found to be increased, whereas HP (50 kDa) was decreased in allergic subjects, confirming mass spectrometric findings ([Fig 3](#)).

DISCUSSION

Nasal mucus acts as a barrier against external pathogens and has antioxidant, antiprotease, and antimicrobial activities.³ A major constituent of nasal mucus are mucins, and approximately 20 mucin genes have been identified in the body.² Their function is not fully understood, but they play a role in antimicrobial and anti-inflammatory responses, as well as in mucociliary clearance in disease when they are overproduced. MUC5, MUC5B, MUC7, and MUC8 are normally expressed in the upper and lower respiratory tracts.^{4,15} MUC5B and MUC7 were identified in the present study, MUC5B with more SC than MUC7. MUC5B was found to be more abundant in mucus from patients with allergic rhinitis without reaching significance. MUC5AC,

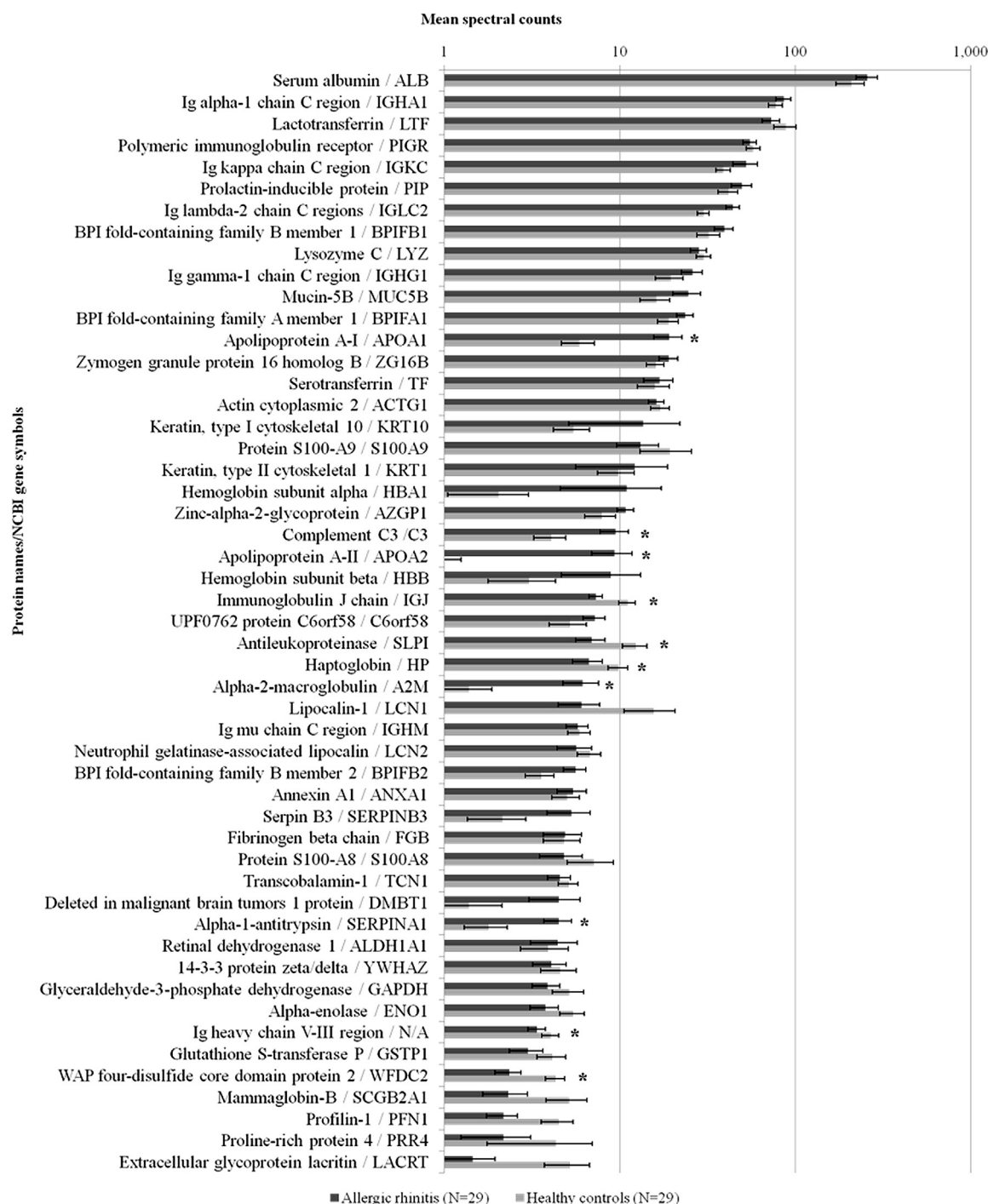


FIG 1. Differences in mean SCs (logarithmic scale) between patients with allergic rhinitis and healthy control subjects for proteins ($n = 51$) with an SC of 4 or greater in either group. Proteins marked with an asterisk are significantly different ($*P < .05$, Mann-Whitney U test).

which is reported to be even more abundant in the airways, could not be detected in our study.⁴ An explanation for the relatively low yield of mucins and other mast cell and eosinophilic proteins might be that insoluble mucous complexes were lost during sample preparation for LC-MS/MS (see the [Methods](#) section in this article's Online Repository). Those mucoclots can be formed through increased glycosylation and sulfation, disulfide cross-linking, and interaction with other proteins. LYZ and LTF, 2

well-known antimicrobial agents of the innate immune system in nasal mucus, were among the most abundantly expressed proteins in the present study in both groups ([Fig 1](#)). Raphael et al¹⁶ found that LYZ and LTF are produced locally in the submucosal nasal glands. Their high abundance comes from cholinergic stimuli, contrary to ALB, higher concentrations of which are found with increased vascular permeability after histamine challenge. Because amounts of LYZ and LTF did not significantly differ

TABLE I. Significantly different proteins between patients with allergic rhinitis and healthy control subjects

Accession no. (UniProt)	Entry name/NCBI gene symbols	P value	Mean SC, AR	SEM, AR	P in N, AR	Mean SC, HC	SEM, HC	P in N, HC	Ratio AR/HC	Mean SC, total	Mean SEM, total
P02652	Apolipoprotein A-II/APOA2	.000	9	2.4	24/29	1	0.3	12/29	9.68	5.16	1.33
P01023	α 2-Macroglobulin/A2M	.002	6	1.4	20/29	1	0.5	11/29	4.45	3.76	0.80
P02647	Apolipoprotein A-I/APOA1	.001	19	3.5	26/29	6	1.3	20/29	3.25	12.52	2.04
P01009	α 1-Antitrypsin/SERPINA1	.003	4	0.8	24/29	2	0.5	16/29	2.50	3.14	0.50
P01024	Complement C3/C3	.026	9	1.7	25/29	4	0.8	21/29	2.32	6.76	1.02
P01766	Ig heavy chain V-III region BRO/NA	.042	3	0.4	28/29	4	0.5	27/29	0.84	3.71	0.30
P00738	Haptoglobin/HP	.008	7	1.3	29/29	10	1.3	29/29	0.68	8.24	0.91
P01591	Immunoglobulin J chain/IGJ	.010	7	0.7	29/29	11	1.2	29/29	0.66	9.19	0.72
P03973	Antileukoprotease/SLPI	.030	7	1.3	27/29	12	2.0	27/29	0.56	9.64	1.22
Q14508	WAP 4-disulfide core domain protein 2/WFDC2	.004	2	0.4	25/29	4	0.5	28/29	0.54	3.33	0.35

Significantly different proteins between patients with allergic rhinitis and healthy controls with a mean SC of 4 or greater are shown. A *P* value of less than .05 was considered significant and was obtained by using the Mann-Whitney *U* test.

AR, Patients with allergic rhinitis; AR/HC, ratio of the mean SC (not rounded) of patients with allergic rhinitis versus healthy control subjects; HC, healthy control subjects; NCBI, National Center for Biotechnology Information; *P* in *N*, presence of protein in number of patients/total number of probands.

between patients with allergic rhinitis and healthy control subjects, we conclude that their secretion is not altered by the disease. Their role in innate immune responses is well known; however, their role in the chronic inflammatory response and the adaptive immune system needs to be determined.¹⁷

These examples demonstrate that proteins contained within the mucus mediate its functions. Changes in the mucus proteome might favor disintegration of its barrier function, leading to direct challenge of the epithelium with various noxa. Few publications exist about the nasal mucus proteome despite its noninvasive properties, which makes it an ideal source for a biomarker search. In one of the first studies on the topic, Casado et al⁵ identified 111 proteins, 42 of which had not been discovered previously. In the current study a total of 267 proteins were discovered, 247 of which were present in healthy control subjects. Mortstedt et al¹⁸ compiled a list of 244 relevant proteins in nasal lavage fluid. Compared with their list, 119 proteins were newly identified in the present study, 12 of which were exclusively present in patients with allergic rhinitis and 18 of which were present in healthy control subjects only (see Table E4 in this article's Online Repository at www.jacionline.org). Of the newly identified proteins, the exclusive presence of angiotensin, apolipoprotein A-4 (APOA4), apolipoprotein B-100 (APOB), complement C5 (C5), filaggrin 2, nonsecretory ribonuclease (RNASE2), and vitronectin (VTN) in patients with allergic rhinitis is striking. Angiotensin is a plasma protein involved in blood pressure regulation and smooth muscle contraction and was shown to trigger bronchial smooth muscle hyperactivity in a mouse model. This is an important finding for the pathogenesis of asthma and might also play a role in allergic rhinitis.¹⁹ APOA4, APOB, APOA1, and APOA2, the latter 2 being significantly more abundant in patients with allergic rhinitis, are lipoproteins that might play an important role in allergic rhinitis. Makino et al²⁰ showed an increase in plasma levels of APOA4 in patients with allergic rhinitis after sublingual immunotherapy compared with those seen after placebo. This also correlated with a reduced symptom score. In this respect an anti-inflammatory effect and decrease in histamine release through APOA4 was observed. C5 is a marker of plasma exudation in immediate reactions to allergen challenge.²¹ Filaggrin gene defects were shown to be associated with epithelial barrier defects and development of atopic dermatitis. Protein

deficiencies could play a role in the development of allergic rhinitis as well. As a structural component of the stratum corneum of the dermis and thus also the nasal vestibule, its presence in mucus from patients with allergic rhinitis in the present study could be another marker of epithelial damage and vascular exudation.²²⁻²⁵ However, the role of filaggrins in the nasal mucosa needs to be further elucidated.²⁵ RNASE2 and VTN are also interesting proteins because RNASE2 is an eosinophil product and VTN is an adhesion molecule to activated eosinophils.^{26,27}

Newly identified proteins present only in healthy control subjects (*n* = 8) are involved in energy metabolism, protein transcription regulation, and nucleic acid synthesis, which might reflect epithelial homeostasis. Of these, 2 proteins with very low levels might be of greater interest: granulin (GRN) and S100-A4. GRN and S100 proteins are important for energy and protein homeostasis, immune responses, and tissue repair. Deficiencies of GRN are linked to neurodegenerative diseases, and deficiencies of S100 proteins, especially S100-A4, are linked to fibrosis²⁸ and tumor invasion.²⁹⁻³¹

To our knowledge, only 1 study has dealt with the nasal mucus proteome and allergic rhinitis.³² However, the study focused on seasonal differences in protein species with different posttranslational modifications by using a 2-dimensional gel-based approach analyzing 20 selected protein species. We used a shotgun proteomics approach with the aim to analyze a more complete set of proteins for a better general overview of the nasal mucus proteome in patients with allergic rhinitis versus healthy control subjects, and thus different methodologies were used and less cases (6 patients and 5 control subjects) were analyzed compared with our study (29 patients and 29 control subjects). Ghafouri et al³² found that cystatin S levels were decreased during the pollen season in patients with allergic rhinitis. We also found lower levels of this cysteine protease in patients with allergic rhinitis, but the difference did not reach significance in our study. We also identified other cysteine proteases, namely caspase-14, cathepsin B, and protein DJ-1. All were identified more often in healthy control subjects but could not be quantified by means of spectral counting (mean SC, <4) because of their low abundance. The latter 2 were only marginally present in patients with allergic rhinitis.

Baraniuk et al³³ discovered enzymatic activity in pollen grains from ragweed, rye grass, and white oak, which rapidly released

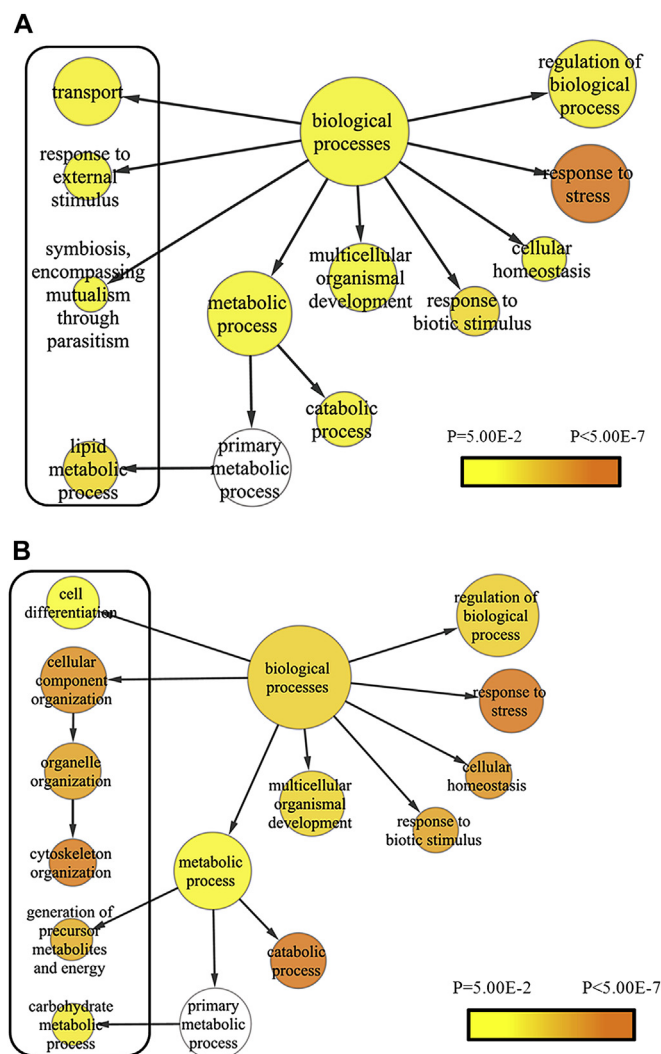


FIG 2. A, Enrichment analysis of biological processes, obtained by using BINGO software, of significantly enriched biological processes of proteins present in patients with allergic rhinitis ($n = 12$) compared with the total human proteome. Nodes surrounded by a *black rectangle* indicate biological processes exclusively found in patients with allergic rhinitis ($n = 4$) compared with healthy control subjects. The *color bar in the right lower quadrant* indicates level of significance from low (yellow) to high (orange). Statistical analysis was performed with a hypergeometric test. A P value of less than .05 was considered significant. **B**, Enrichment analysis of biological processes, obtained by using BINGO software, of significantly enriched biological processes of proteins present in healthy control subjects ($n = 14$) compared with the total human proteome. Nodes surrounded by a *black rectangle* indicate biological processes exclusively found in healthy control subjects ($n = 6$) compared with patients with allergic rhinitis. The *color bar in the right lower quadrant* indicates level of significance. Statistical analysis was performed with a hypergeometric test. A P value of less than .05 was considered significant.

esterases. Raftery et al³⁴ used mass spectrometry to identify proteinase activity in Kentucky blue grass, rye grass, and Bermuda grass. If pollen proteases are not sufficiently inhibited by the endogenous antiproteases or proteinase inhibitors, this could lead to epithelial damage up to degradation of tight junctions⁹ and penetration and transport of allergens across the epithelium.^{7,8,35} Next to epithelial dysfunction, pollen proteases induce proinflammatory cytokines and T-cell responses. The presence of cysteine protease activity in major pollen grains was shown,³⁶ and

Kamijo et al³⁷ recently demonstrated that cysteine proteases induce IL-33, eosinophilia, and antigen production. Blocking cysteine protease activity by a cysteine protease inhibitor (E64) resulted in decreased lung eosinophilia and serum antigen production. This suggests a protease-dependent inflammatory response contrary to conformational changes, as tested by means of heat denaturation of the protein.

A2M is the only significantly increased antiprotease in mucus from patients with allergic rhinitis with potential for inhibition of all 4 classes of proteinases (serine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases). It traps the enzyme and decreases its proteolytic activity for high-weight substrates by hydrolyzing a thioester bond and covalent binding to the protease.³⁸⁻⁴¹ Next to A2M, another proteinase inhibitor, SERPINA1, was significantly more abundant in patients with allergic rhinitis. However, SERPINA1 is a serine protease inhibitor of the serpin family,⁴²⁻⁴⁴ and it was shown that proteases released from pollen grains are not inhibited by serine protease inhibitors.⁸ Its primary target is elastase, but it also moderately inhibits plasmin and thrombin with irreversible inhibition of trypsin, chymotrypsin, and plasminogen activator. However, other serine proteinase inhibitors, such as WFDC2 and SLPI, were significantly less abundant in our patients with allergic rhinitis. WFDC2 regulates the proliferation of epithelial cells by inhibiting serine proteases to degrade laminin and suppressing the mitogen-activated protein kinase pathway in the cell cycle.^{45,46} SLPI is a secreted, acid-stable protease inhibitor with strong affinities for trypsin, chymotrypsin, elastase, and cathepsin G.⁴⁷⁻⁴⁹ WFDC2 and SLPI protect the epithelium and prevent damage to mucosal tissues through protease inhibition, such as elastases, which cannot degrade laminin. Furthermore, these proteins regulate epithelial proliferation through mitogen-activated protein kinase pathway inhibition and have immunomodulatory effects in the mucosa.^{46,49-53} Thus the epithelium is protected through these proteins in the mucus, and allergen penetration and presentation might be inhibited in the mucus. The observed overall distribution suggests an imbalance between cysteine proteases and particularly serine proteases/antiproteases in patients with allergic rhinitis.

On the other hand, A2M is an index for increased epithelial permeability and intraluminal plasma exudation, which is induced in patients with allergic rhinitis. This is also true for ALB and eosinophilic cationic protein, as published earlier by Svensson et al,⁵⁴ who described vascular exudation of these proteins as a marker for long-lasting allergic disease and allergen challenge. Although there was no significant findings in the present study, ALB and eosinophilic cationic protein levels were increased in patients with allergic rhinitis. Native mucus protein concentrations did not differ significantly between patients with allergic rhinitis and healthy control subjects. Yet of the proteins exclusively present in either group, 10 (50%) of 20 in patients with allergic rhinitis and only 5 (20%) of 25 in healthy control subjects were plasma proteins. In patients with allergic rhinitis 6 (30%) of 20 and in healthy control subjects 7 (28%) of 25 were of epithelial and glandular origin. This supports the theory of increased epithelial permeability and plasma exudation of proteins in patients with allergic rhinitis, which could be seen as a defense mechanism.^{40,55}

APOA1 and APOA2 levels were also significantly increased in mucus from patients with allergic rhinitis. They are involved in lipid metabolism and signaling and are present in high-density

TABLE II. Significantly enriched biological processes in patients with allergic rhinitis only with corresponding proteins involved (presented in National Center for Biotechnology Information gene symbols)

Allergic rhinitis (biological processes)	Proteins involved in biological processes							
Transport	AKR1C1	ALB	ANXA1	APOA1	APOA2	APOB	APOH	CP
	CRYM	DMBT1	AHSG	HBA1	HBB	HPX	KRT18	KN1G
	MYH9	NPC2	PLTP	HNRNPA2B1	PSAP	SELENBP1	F2	TF
Response to external stimulus	TTR	GC						
	A2M	ALB	SERPINC1	AZU1	C5	IGFBP7	JUP	PON1
	RNASE2							
Symbiosis, encompassing mutualism through parasitism	ALB	CAMP	CTSG					
Lipid metabolic process	ADH7	AKR1C1	ANXA1	APOA1	APOA2	APOB	APOH	ASAHI
	ECH1	ALOX15	PLTP	PON1	PSAP	AZGP1		

TABLE III. Significantly enriched biological processes in healthy control subjects only with corresponding proteins involved (presented in National Center for Biotechnology Information gene symbols)

Healthy control subjects (biological processes)	Proteins involved in biological processes							
Cell differentiation	ACTR3	BASP1	CASP14	CFL1	EZR	FLG	G6PD	ARHGDIA
	GSN	LTA4H	LMNA	MMP9	PLA2G2A	PEBP1	S100A4	S100A6
	SOD1	SPRR1A	SPRR1B	SPRR3	TTN	TYMP	CXCL17	
Cellular component organization	YWHAZ	ACTN4	ALDOA	ANXA2	ANXA3	ANXA5	ARF1	ATP5B
	CAP1	CAT	CTSD	CDA	CLTC	CFL1	CORO1A	CST3
	ELANE	EZR	FGA	ARHGDIB	GSN	HIST1H1D	HIST1H4A	HSP90AA1
	KRT9	LMNA	MMP9	PLA2G2A	PDS5A	LCP1	PFN1	RUVBL1
	S100A4	S100A6	S100A9	SOD1	TUBA1B	TUBB4B	VCP	TTN
Organelle organization	TPPP3	TMSB4X	TYMP					
	YWHAZ	ACTN4	ALDOA	ARF1	CAP1	CTSD	CLTC	CFL1
	CORO1A	EZR	ARHGDIB	GSN	HIST1H1D	HIST1H4A	HSP90AA1	KRT9
	LMNA	PDS5A	LCP1	PFN1	RUVBL1	S100A9	SOD1	TUBA1B
	TTN	TPPP3	TMSB4X	TYMP				
Cytoskeleton organization	ACTN4	ALDOA	CAP1	CFL1	CORO1A	EZR	ARHGDIB	GSN
	KRT9	LCP1	PFN1	S100A9	SOD1	TUBA1B	TTN	TPPP3
	TMSB4X							
Generation of precursor metabolites and energy	ALDOA	ATP5B	CAT	ENO1	GAPDH	GPI	PKM	PGK1
	PYGB	TXN	TPI1	TXNRD1				
Carbohydrate metabolic process	PGD	ALDOA	SORD	ENO1	GAPDH	G6PD	GPI	PKM
	PGK1	PYGB	TALDO1	TPI1				

lipoproteins and chylomicrons.⁵⁶⁻⁵⁸ APOA1 and APOA2 were found to downregulate neutrophil function.⁵⁹ APOA1 suppresses bacterial growth and acts as an anti-inflammatory agent. Its upregulation in patients with allergic rhinitis could reflect the recurring inflammation of the nasal mucosa in patients with allergic rhinitis. Its role in the chronic immune response is more complex. Nagel et al⁶⁰ suggested a relation of increased APOA1 levels to atopy and asthma in schoolchildren. There is no evidence for apolipoprotein production in the nasal mucosa, and their higher abundance in patients with allergic rhinitis reflects a higher vascular permeability. However, plasma levels of APOA1 and APOA2 have not been measured in this study, and further experiments are required to determine their role in allergic rhinitis.

APOA1 and APOA2, as well as other lipoproteins, seem to be involved in antimicrobial defense through LPS binding and suppression of bacterial growth.^{61,62} The mechanisms of LPS binding are not clear, yet electrostatic interactions between the ϵ -amino groups of lysine side chains to LPS are proposed.⁶² This antimicrobial activity is similar to the PLUNC protein, which was shown to be able to neutralize LPS.⁶³⁻⁶⁵ Two PLUNC family members were identified (ie, BPI fold-containing family

A B member 1 [BPIFA1] and BPIFB1) and found to be more abundant in patients with allergic rhinitis without reaching significance. BPIFA1⁶⁶ is also involved in innate mucosal defense, probably through inhibition of bacterial biofilm formation. Other identified lipocalin family proteins also have overlapping antimicrobial functions: they sequester enterobactin, disabling it to retrieve iron, which is essential for bacteria to grow.⁶⁷⁻⁶⁹ Its induction is many-fold by Toll-like receptor-dependent signaling and subsequent T-cell activation and T-cell cytokine pathways. In the present study lipocalin 1, neutrophil gelatinase-associated lipocalin, and lipocalin 15 were identified. Only lipocalin 1 and neutrophil gelatinase-associated lipocalin were abundant enough for relative quantitation (mean SC ≥ 4), but their lower abundance in patients with allergic rhinitis did not reach significance. Despite their anti-inflammatory effect in acute inflammation, sustained upregulation did not protect against allergic airway inflammation, as stated by Dittrich et al.⁶⁹

C3 was significantly more abundant and C5 was newly identified in mucus from patients with allergic rhinitis. They play a role in the inflammatory process of allergic rhinitis through activation of the complement system.⁷⁰ They are markers of

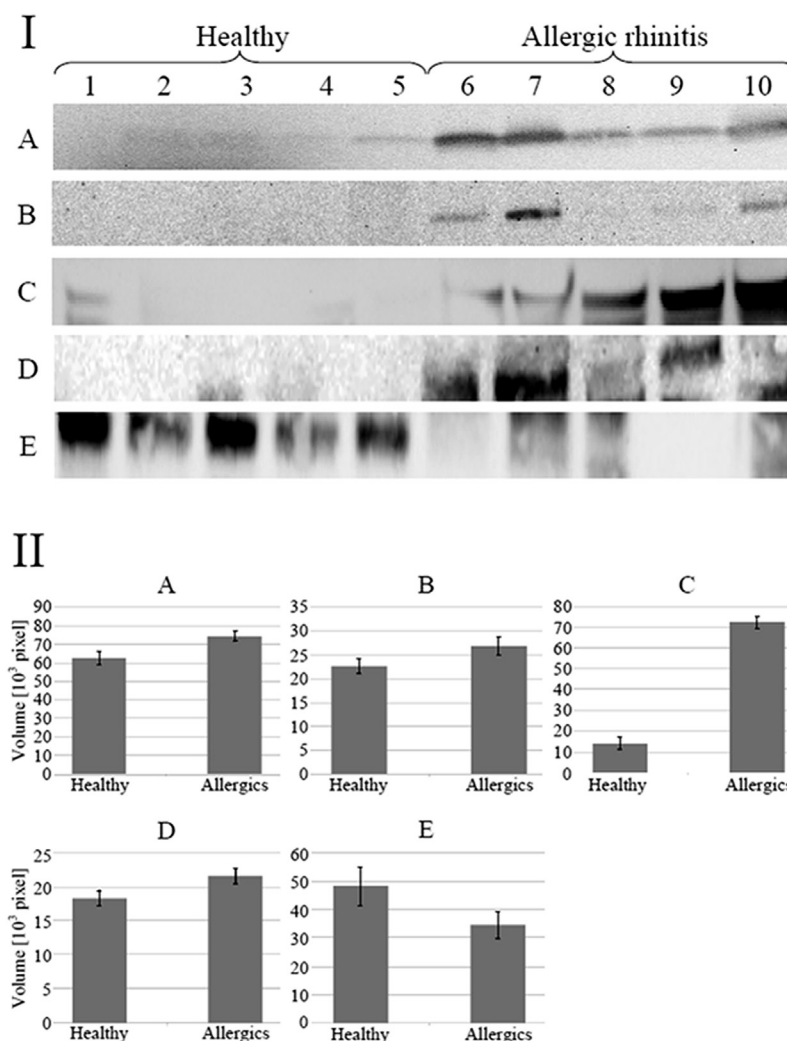


FIG 3. Western blot analysis of selected proteins. Five proteins, APOA2 (A), A2M (B), SERPINA1 (C), C3 (D), and HP (E), were analyzed. Panel I shows the respective immunoblots of 5 healthy control subjects and 5 patients with allergic rhinitis. Estimated molecular weights in comparison with a protein standard were 12 kDa for APOA2, 160 kDa for A2M, 55 kDa for SERPINA1, 120 kDa for C3, and 50 kDa for HP. Panel II shows the densitometric analysis of the immunoblots. Means and SEMs are compared in healthy subjects versus allergic patients for each protein.

plasma exudation in acute reactions to allergen challenge.²¹ The C3 derivative C3a anaphylatoxin is a mediator of the local inflammatory process. Its activity is mediated through expression of its receptor in nasal epithelial cells.⁷¹ It induces smooth muscle contraction and increases vascular permeability and histamine release from mast cells and basophils, causing mucosal swelling and exudation.⁷¹ Patients with a C3 deficiency have recurrent, severe pyogenic infections because pathogens are not opsonized. Some patients might also have autoimmune disorders, such as arthralgia and vasculitic rashes, lupus-like syndrome, and membranoproliferative glomerulonephritis. These facts underline the importance of mucus as a first-line barrier and site of immune reaction in patients with allergic rhinitis. Complement activation reflects the acute-phase inflammatory reaction of mucus toward allergen challenge. A consecutive increase of epithelial permeability underlines the theory of transepithelial allergen penetration, with subsequent presentation to immune cells.⁷ When biological processes were analyzed, healthy control subjects

showed enrichment of generation of precursor metabolites and energy, as well as carbohydrate metabolic process, which was not detected in patients with allergic rhinitis. Obviously energy metabolism is impaired in the disease state. This could cause epithelial cell death through “starving” cells, which consequently hampers barrier function. Less energy metabolites could also cause reduced mucociliary transport and clearance. The allergens might be cleared less rapidly, leaving more time for allergen presentation and processing at the epithelium. Other biological processes in normal mucus were cellular component organization, organelle organization, and cytoskeletal organization, which are not seen in patients with allergic rhinitis. These processes are important in normal mucosa for epithelial integrity and also for intracellular and transcellular transport. Again, this addresses the importance of tight junctions. If intracellular adhesion is reduced, allergens could pass more easily through the epithelium or even through cells not facing exocytosis. Cell differentiation was also not seen in patients with allergic rhinitis. However,

ciliated epithelial cells are highly differentiated and fulfill their function in protecting the mucosa. Biological processes in normal mucosa reflect the higher integrity of the epithelium and nasal mucus serving as a primary barrier. To hypothesize, this might not allow proper regeneration but allow allergens to damage the mucosa, pass through the epithelium, and subsequently cause inflammation. Interestingly, the lipid metabolic process was significantly enriched in patients with allergic rhinitis, underlining the important role of apolipoproteins in inflammatory processes.

A shortcoming of this study is the lack of a seasonal analysis, and thus further investigation will be targeted toward intra-individual seasonal changes and analysis of mucus proteome differences in patients with allergic rhinitis and healthy control subjects over the year.

Nasal mucus is the first line of defense and is challenged by various allergens and pathogens. On a proteome basis, mucus from patients with allergic rhinitis shows a decrease in anti-protease activity and an enhanced innate immune response. The imbalance of cysteine proteases and antiproteases in mucus from patients with allergic rhinitis could affect the epithelial barrier if pollen proteases are released on mucosal surfaces, breaking up tight junctions. Other key proteins, such as apolipoproteins, in mucus might create a favorable environment for cellular and humoral immune reactions, perpetuating an inflammatory state of the mucosa. Proteins in healthy control subjects showed protective and anti-inflammatory effects promoting mucus and mucosal homeostasis. Understanding the mucus proteome further sheds light on its role in pathophysiology and lays the groundwork for the identification of biomarkers in patients with allergic rhinitis.

Clinical implications: Nasal mucus is the first-line defense barrier against various pathogens and allergens. The focus of this study lies on its proteome and the understanding of its pathophysiological role in allergic rhinitis.

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Methods supplement

Sample preparation: After defrosting 500 µl phosphate buffered saline were added to the samples, thoroughly mixed and centrifuged at 12000 g for 5 min to remove insoluble particles. Protein content was estimated by Bradford assay (Bio-Rad, Vienna, Austria). 50 µg of protein was precipitated with >10 volumes of acetone at -20°C overnight. Protein was collected by centrifugation at 10000 rpm for 10 min, resolubilised in 35 µl 6 M urea, reduced with 5 mM DTT for 20 min by shaking at 550 rpm at 56°C and alkylated with 10 mM IAA by shaking at 550rpm at RT for 15 min. The samples were diluted to 0.9 M urea with 155 µl 100 mM ammonium bicarbonate. Protein was digested with 1 µg trypsin by shaking at 550 rpm over night at 37°C. Samples were acidified with 6 µl 5 % formic acid. Completion of digest was controlled by 4-12% SDS-PAGE of 2 µg aliquots of digested versus undigested samples and silver staining. 2.5 µg of protein digest was filled up to 50 µl with 0.1% formic acid for proteomic analysis.

Mass spectrometric analysis: 40 µl, i.e. 2 µg, were separated by nano-HPLC on an Agilent (Vienna, Austria) 1200 system equipped with a Zorbax 300SB-C18, 5µm, 5 x 0.3mm enrichment column and a Zorbax 300SB-C18, 3.5 µm, 150 x 0.075 mm nanocolumn. Samples were injected and concentrated on the enrichment column for 6 min using 0.1 % formic acid as isocratic solvent at a flow rate of 20 µL/min. The column was then switched into the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 300 nL/min and separated using the following gradient: solvent A: water, 0.1% formic acid; solvent B: acetonitril/water 80/20, 0.1% formic acid; 0-10min: 10% B; 10-130min 10-60% B, 130-132min 60-95% B, 132-140min 95% B, 140-140.01 min 95-10%. The sample was ionized in the nanospray source equipped with nanospray tips (PicoTip™ Stock# FS360-75-15-D-20, Coating: 1P-4P, 15+/- 1µm Emitter, New Objective) and analyzed in a Thermo Scientific (Vienna, Austria) LTQ-FT mass spectrometer in positive ion mode by alternating full scan

MS (m/z 400 to 2000) in the ICR cell and MS/MS by CID of the 5 most intense peaks in the ion trap with dynamic exclusion enabled (for a duration of 10s).

Mass spectrometric data analysis: The LC-MS/MS data were analyzed by searching the human SwissProt public database (downloaded on March 10th 2012) with Spectrum Mill Rev. A.03.03.078 (Agilent, Vienna, Austria) and Proteome Discoverer 1.3 (Thermo Scientific, Vienna, Austria) using Mascot 2.2 (MatrixScience, London, UK). Detailed settings: Enzyme: trypsin, max. missed cleavage sites: 2, carbamidomethylation on lysine as fixed modification, oxidised methionine as variable modification, maximum precursor charge 3; precursor mass tolerance +/- 5 ppm, product mass tolerance +/- 0.7 Da; acceptance parameters were 2 or more identified distinct peptides after automatic validation (Mascot: decoy search, FDR < 5%; Spectrum Mill: for precursor charge of 2: score threshold was 6.0, %SPI threshold was 60.0, Fwd-Rev score threshold was 2.0 and rank 1-2 score threshold was 2.0, for precursor charge of 1: score threshold was 6.0, %SPI threshold was 70.0, Fwd-Rev score threshold was 2.0 and rank 1-2 score threshold was 2.0, for precursor charge of 3: score threshold was 8.0, %SPI threshold was 70.0, Fwd-Rev score threshold was 2.0 and rank 1-2 score threshold was 2.0).

TableE4a: Newly identified proteins present exclusively in allergic rhinitis patients (A) presented with UniProt accession numbers and NCBI Gene Symbols.

Acc. No.	NCBI Gene Symbols	Group
P01019	AGT	A
P01031	C5	A
P04004	VTN	A
P04114	APOB	A
P06727	APOA4	A
P10153	RNASE2	A
P13010	XRCC5	A
Q13011	ECH1	A
Q13510	ASAH1	A
Q14894	CRYM	A
Q16270	IGFBP7	A
Q5D862	FLG2	A

Acc.No.= UniProt accession number

A = proteins present in allergic rhinitis mucus only

TableE4b: Newly identified proteins present exclusively in healthy controls (H) presented with UniProt accession numbers and NCBI Gene Symbols.

Acc. No.	NCBI Gene Symbols	Group
P01714	N/A*	H
P04196	HRG	H
P05387	RPLP2	H
P06753	TPM3	H
P07108	DBI	H
P07910	HNRNPC	H
P08758	ANXA5	H
P11413	G6PD	H
P12277	CKB	H
P20618	PSMB1	H
P26447	S100A4	H
P28799	GRN	H
P32320	CDA	H
P52565	ARHGDIA	H
P61160	ACTR2	H
P61978	HNRNPK	H
Q16881	TXNRD1	H
Q9Y265	RUVBL1	H

Acc.No.= UniProt accession number

H = proteins present healthy control mucus only

* = Ig lambda chain V-III region SH (no NCBI Gene Symbol available)

