

Apolipoproteins Have a Potential Role in Nasal Mucus of Allergic Rhinitis Patients: A Proteomic Study

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Objectives/Hypothesis: Nasal mucus is a defense barrier against aeroallergens. We recently found apolipoproteins to be elevated in the nasal mucus of allergic rhinitis patients. Apolipoproteins are involved in lipid metabolism, have immunomodulatory properties, and may represent interesting novel biomarkers. This study aims to validate our findings and analyze whether the increased abundance of apolipoproteins in nasal mucus is a local or systemic phenomenon in allergic rhinitis.

Study Design: Prospective controlled trial.

Methods: Nasal mucus of allergic rhinitis patients (n = 10) and healthy controls (n = 12) was collected, tryptically digested, and analyzed by LC-MS/MS. Areas under the curve (AUCs) of the total peptides identified and matched to apolipoproteins were used to compare relative protein abundances of the same protein between groups.

Results: In a total of 389 identified proteins in nasal mucus, apolipoproteins A-I, A-II, A-IV, and B 100 were detected. Apolipoprotein A-I (mean normalized AUC 1.49% [SEM = 0.5] vs. 0.42% [SEM = 0.2]) and A-II (mean normalized AUC 0.47% [SEM = 0.2] vs. 0.05% [SEM = 0.02]) were significantly more abundant in allergic rhinitis patients than controls (3.6-fold and 9.4-fold, respectively). Apolipoprotein A-IV (mean normalized AUC = 0.01%) and B-100 (mean normalized AUC = 0.02%) were each detected in only one allergic rhinitis patient out of 10. Myeloperoxidase was detected with a mean normalized AUC of 0.06% (SEM = 0.03) in allergic rhinitis patients and 0.18% (SEM = 0.08) in healthy controls without reaching significance.

Conclusion: This study confirms the significantly higher abundance of apolipoproteins A-I and A-II in allergic rhinitis mucus. Their release seems to be triggered by local mechanisms as an antiinflammatory response to allergens.

Key Words: Apolipoproteins, allergic rhinitis, mass spectrometry, nasal mucus, proteome.

Level of Evidence: 3b.

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INTRODUCTION

Allergic rhinitis is a global health problem significantly impairing patients' quality of life. The disease affects up to 40%¹ of the population, including all age groups, at great expense to the health care system. Despite a variety of therapies from antihistamines to specific immunotherapy, improving the individual patient's situation still challenges the physician.²

Together with the epithelial lining, nasal mucus is the first-line defense barrier against aeroallergens causing allergic rhinitis. Nasal mucus proteins in particular have potential as novel biomarkers that could open new therapeutic strategies. Since nasal mucus contains hundreds of different proteins, proteomic techniques could be applied to meet the challenge of identifying key proteins or protein groups.³⁻⁶

Apolipoproteins have been investigated in recent years as interesting potential biomarkers for inflammatory diseases because they are not only involved in lipid metabolism but also have immunomodulatory properties. They influence cytokine and chemokine release as well as cell adhesion molecule expression.^{7,8} Especially in asthma, apolipoproteins were found to restore bronchoalveolar epithelial integrity, to reduce goblet cell hyperplasia and mucin expression, and to modulate airway hyperreactivity.^{9,10} Granulocyte infiltration is also reduced.¹¹ Although a majority of studies attribute anti-inflammatory mechanisms to apolipoproteins, they could also act as proinflammatory molecules.⁸ This may be the result of posttranslational change through oxidative mechanisms.

Little is known about the role of apolipoproteins in allergic rhinitis, although they were discovered in healthy adults' nasal fluid¹² and found to be elevated in allergic rhinitis patients in our previous study.³⁻⁶ Here,

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TABLE I.
Demographic Data Including Age, Gender Distribution, Sensitization Pattern, Symptoms, and Plasma IgE of the Study Collective.

Parameter	Healthy Controls	Allergic Rhino-Conjunctivitis
Number of patients	12	10
Demographics		
Mean age [years] (SD)	36.3 (10.1)	30.4 (8.4)
Women [%]	40%	75%
Clinically relevant positive SPT		
Alder/hazel/birch pollen	0	8
Grass pollen mix	0	9
Ragweed pollen	0	3
Symptoms during pollen season		
Allergic rhinoconjunctivitis	0	10
Allergic rhinoconjunctivitis and asthma	0	0
Total IgE [kU/l] (SEM)	42 (37.5)	136.1 (122.1)

SD = standard deviation; SEM = standard error of mean; SPT = skin prick test.

we validated our finding with a another label-free proteomics approach (based on areas under the curve of extracted ion chromatograms instead of spectral counting) and investigated the source of elevated apolipoproteins in allergic rhinitis by analyzing abundances of selected apolipoproteins in nasal mucus as compared to plasma concentrations in order to determine whether the high abundance in mucus is due to plasma exudation or a local mechanism.

MATERIALS AND METHODS

Patients

Twenty-two individuals (9 male, 13 female) with a mean age of 34 years (range: 19–61 years) were included in a study group comprising 10 (45%) allergic rhinitis patients and 12 (55%) healthy controls. Allergy status was verified by skin prick tests (SPT) (Allergopharma GmbH & Co. KG, Reinbek, Germany) and specific IgE (ImmunoCAP; Thermo Fisher Scientific Inc., Vienna, Austria) in all patients and controls. Patients sensitized to house dust mite or animals only were excluded to avoid bias due to small sample size (Table I). Thus, only patients sensitized to pollen and also showing symptoms during the pollen season were considered for evaluation. Patients with acute and/or chronic sinusitis as defined by the EPOS¹³ guidelines were also excluded—as were patients with malignant tumors and any infectious or cardiopulmonary disease—or who had been treated with systemic or topical drugs including antihistamines, corticosteroids, antibiotics, antifungals, or any other immunomodulatory drugs in the 4 weeks prior to the study. The same exclusion criteria applied to the controls, who were healthy volunteers recruited from the hospital staff. Informed consent was obtained from all participants (allergics and controls) before enrolment. The study was approved by the institutional review board of the Medical University of Graz.

Sample Collection

During pollen season (with clinical symptoms present in allergic rhinitis patients) nasal mucus was collected with a special suction device (Sinus Secretion Collector; Medtronic Xomed

Inc., Jacksonville, FL). Healthy controls' samples were collected on the same day as allergic rhinitis patients' samples. Without previous interventions (decongestants, 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. The mucus volume obtained was approximately equal in both groups. Then, mucus was deep-frozen at -92°C before processing for LC-MS/MS mass spectrometry. Blood samples were taken, and plasma levels of

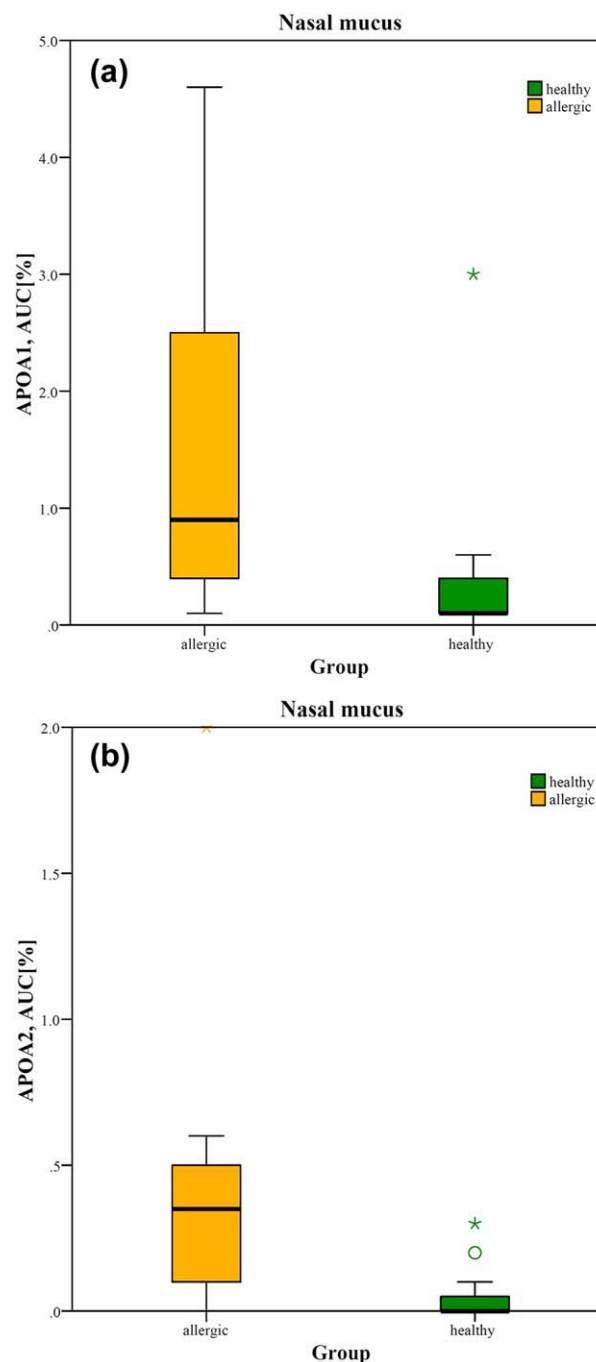


Fig. 1. **a.** Mean normalized areas under the curve (AUC [%], y-axis) of ApoA-I in allergic rhinitis patients and healthy controls in nasal mucus. **b.** Mean normalized areas under the curve (AUC [%], y-axis) of ApoA-II in allergic rhinitis patients and healthy controls in nasal mucus. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

TABLE II.
Mean AUCs of ApoA-I, ApoA-II, and MPO in Nasal Mucus as Well As Plasma Concentrations of ApoA-I and ApoB.

Protein NCBI Gene Symbol	Accession No.	Group			
		Allergic		Healthy	
		Mean Normalized AUC [%]	SEM [%]	Mean Normalized AUC [%]	SEM [%]
APOA1	P02647	1.49	.46	.42	.24
APOA2	P02652	.47	.18	.05	.03
MPO	P05164	.06	.03	.18	.08
		mean plasma concentration [mg/dl]	SEM [mg/dl]	mean plasma concentration [mg/dl]	SEM [mg/dl]
APOA1	P02647	187.67	13.65	179	10.41
APOB	P04114	87.44	3.75	77.64	5.70

AUC = areas under the curve; SEM = standard error of mean; ApoA-I = Apolipoprotein A-I; ApoA-II = Apolipoprotein A-II; ApoB = Apolipoprotein B; MPO = Myeloperoxidase.

apolipoprotein A-I as the main protein component of high density lipoprotein (HDL) and apolipoprotein B as the main component of low density lipoprotein,¹⁴ were determined by standard immunonephelometry with antiserum against Apolipoprotein A-I and B (BN System; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Correlation between mucus abundance and plasma concentration of Apolipoprotein A-I was determined to see whether abundance changes are due to plasma exudation or a local mechanism in equal mucus and physiologic plasma volumes. Additionally, plasma concentration of Apolipoprotein B was determined to verify normal lipid and lipoprotein status in patients and controls.

Sample Preparation

After defrosting, 500- μ l phosphate buffered saline were added to the samples, thoroughly mixed, and centrifuged at 12,000 g for 5 minutes to remove insoluble particles. Protein content was estimated by Bradford assay (Bio-Rad, Vienna, Austria). Fifty μ g of protein was precipitated with > 10 volumes of acetone at -20°C overnight. Protein was collected by centrifugation at 10,000 rpm for 10 minutes, resolubilized in 0.35 μ l 6 M urea, reduced with 5 mM dithiothreitol for 20 minutes by shaking at 550 rpm at 56°C , and alkylated with 10 mM iodoacetamide by shaking at 550 rpm at RT for 15 minutes. The samples were diluted to 0.9 M urea with 155 μ l 100 mM ammonium bicarbonate. Protein was digested with 1 μ g trypsin by shaking at 550 rpm overnight at 37°C . Samples were acidified with 6 μ l 5% formic acid. Completion of digestion was controlled by 4% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis of 2 μ g aliquots of digested versus undigested samples and silver staining; 2.5 μ g of protein digest was filled up to 50 μ l with 0.1% formic acid for proteomic analysis.

Mass Spectrometric Analysis

By nano-HPLC, 40 μ l (i.e., 2 μ g) were separated by nano-HPLC on an Agilent (Vienna, Austria) 1200 system equipped with a Zorbax 300SB-C18, 5 μ m, 5×0.3 -mm enrichment column and a Zorbax 300SB-C18, 3.5 μ m, 150×0.075 mm nanocolumn. Samples were injected and concentrated on the enrichment column for 6 minutes using 0.1 % formic acid as isocratic solvent at a flow rate of 20 μ L/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/minutes

and separated using the following gradient: solvent A) water, 0.1% formic acid; solvent B) acetonitril/water 80/20, 0.1% formic acid (0–10 min 10% B; 10–130 min 10%–60% B; 130–132 min 60%–95% B, 132–140 min 95% B, 140–140.01 min 95% to 10%). The sample was ionized with nanospray tips in the nanospray source (PicoTipTM Stock FS360-75-15-D-20; Coating: 1P-4P, 15 ± 1 μ m 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 400–2000) in the ion cyclotron resonance cell and MS/MS by collision induced dissociation of the five most intense peaks in the ion trap with dynamic exclusion enabled (for a duration of 10 sec).

Proteomic experiments were performed according to minimum information about a proteomic experiment.¹⁵

Data Analysis

The LC-MS/MS data were analyzed by searching the human SwissProt public database (downloaded on March 10, 2012) with Proteome Discoverer 1.4 (Thermo Scientific) and Mascot 2.2 (MatrixScience, London, UK). Detailed settings: enzyme (trypsin); maximum missed cleavage sites (2); N-terminus (hydrogen); C-terminus (free acid, carbamidomethylation on lysine as fixed modification, oxidized methionine as variable modification); maximum precursor charge 3 (precursor mass tolerance $\pm 0.05\text{Da}$, product mass tolerance $\pm 0.7\text{Da}$); and acceptance parameters were 2 or more identified distinct peptides after automatic validation (decoy search, FDR < 5%). Identified proteins were annotated using data from Uniprot (www.uniprot.org). Areas under the curve (AUCs) (i.e., mean areas of extracted ion chromatograms of the individual peptides matched to a protein) normalized on the total AUC of all proteins in each sample were used to compare relative protein abundances of the same protein between groups.¹⁶ Data are reported as means and standard errors of mean (SEM). Statistical analysis by Mann Whitney U test and Pearson correlation coefficient were performed with SPSS 18.0 software (Chicago, IL). A *P* value of < 0.05 was considered significant.

RESULTS

Nasal Mucus

In total, 389 proteins in nasal mucus were identified, and apolipoprotein A-I, A-II, A-IV and B-100 could

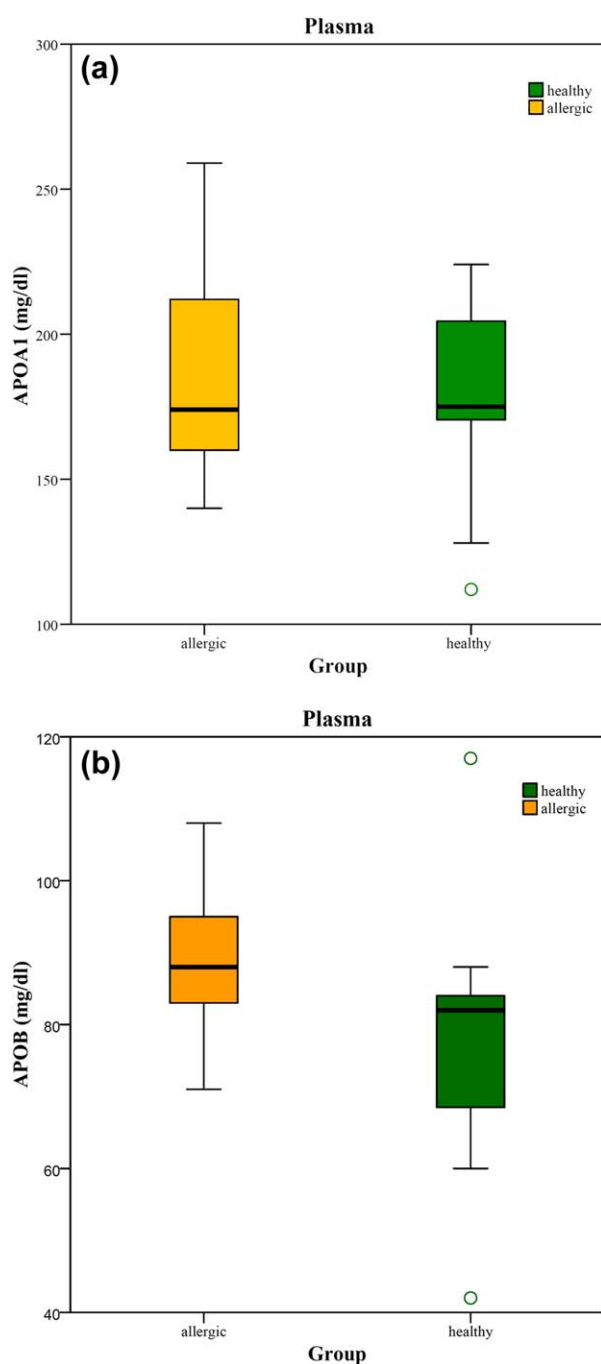


Fig. 2. a. Mean plasma concentrations (mg/dl) of ApoA-I in allergic rhinitis patients and healthy controls. b. Mean plasma concentrations (mg/dl) of ApoB in allergic rhinitis patients and healthy controls. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

be detected. Apolipoprotein A-I was present in 10/10 allergic rhinitis patients and 10/12 healthy controls. Apolipoprotein A-II was present in nine of 10 allergic rhinitis patients and three of 12 healthy controls. Apolipoprotein A-I (mean normalized AUC 1.49% [SEM = 0.5] vs. 0.42% [SEM = 0.2]) and A-II (mean normalized AUC 0.47% [SEM = 0.2] vs. 0.05% [SEM = 0.02]) were significantly more abundant in allergic rhinitis patients than

in healthy controls (3.6-fold and 9.4-fold, respectively) (Fig. 1, Table II). Apolipoprotein A-IV (mean normalized AUC = 0.01%) and B-100 (mean normalized AUC = 0.02%) were each detected in only one allergic rhinitis patient out of 10. Myeloperoxidase was present in three of 10 allergic rhinitis patients and six of 12 healthy controls with a mean normalized AUC of 0.06% (SEM = 0.03) in allergic rhinitis patients and 0.18% (SEM = 0.08) in healthy controls without reaching significance.

Plasma

Mean plasma concentration of apolipoprotein A-I was 188.8 mg/dl (SEM = 10.2) in allergic rhinitis patients and 178 mg/dl (SEM = 5.5) in healthy controls ($P = 0.8$). Mean plasma concentration of apolipoprotein-B was 89.5 mg/dl (SEM = 3.9) in allergic rhinitis patients and 84.5 mg/dl (SEM = 4.9) in healthy controls ($P = 0.4$) (Fig. 2, Table II). Plasma levels of Apo A-I and Apo-B were within the physiologic range in both groups.

The corresponding changes in plasma concentration of Apo A-I in allergic rhinitis patients ($r = 0.6$) and healthy controls ($r = 0.3$) did not correlate significantly to the abundance changes in the mucus (Fig. 3).

DISCUSSION

Nasal mucus and nasal epithelium are the first-line defense barriers that allergens encounter.^{1,2,17} The subsequent allergen presentation to dendritic cells leads to the well-known immune reactions and clinical symptoms of allergic rhinitis. Proteins in nasal mucus are particularly important biomarkers and show different abundance levels in allergic rhinitis patients and healthy controls.³ In recent years, apolipoproteins have received increasing attention, not only for their role lipid metabolism, but also for their immunomodulatory effects. Anti-inflammatory properties have also been attributed to them, especially in asthma.^{9–11}

Few studies, however, deal with the impact of apolipoproteins in allergic rhinitis. Makino et al.¹⁸ investigated apolipoprotein A-IV in serum of patients undergoing allergen-specific sublingual immunotherapy. They found that ApoA-IV was significantly higher in the treated group than in the placebo group, and its higher abundance correlated with lower symptom score in therapy responders. Moreover, basophil histamine release in serum was reduced after administration of ApoA-IV in vitro, which was not the case for apolipoprotein A-I and E.

Do et al.¹⁹ discovered lipoproteins in nasal lavage fluid. Ghafouri et al.^{4,19} detected apolipoprotein A-I in nasal fluid, whereas apolipoprotein B-100 and E could not be found. In our previous study,³ ApoA-I and ApoA-II were significantly more abundant in allergic rhinitis patients. The -fold changes between allergic rhinitis patients and healthy controls in our previous study (apolipoprotein A-I, 3.2-fold; apolipoprotein A II, 9.7-fold) are similar to our present findings (3.6-fold and 9.4-fold, respectively), thus validating our results. In our present study, we took mucus samples in season when protein abundance differences between allergic rhinitis patients

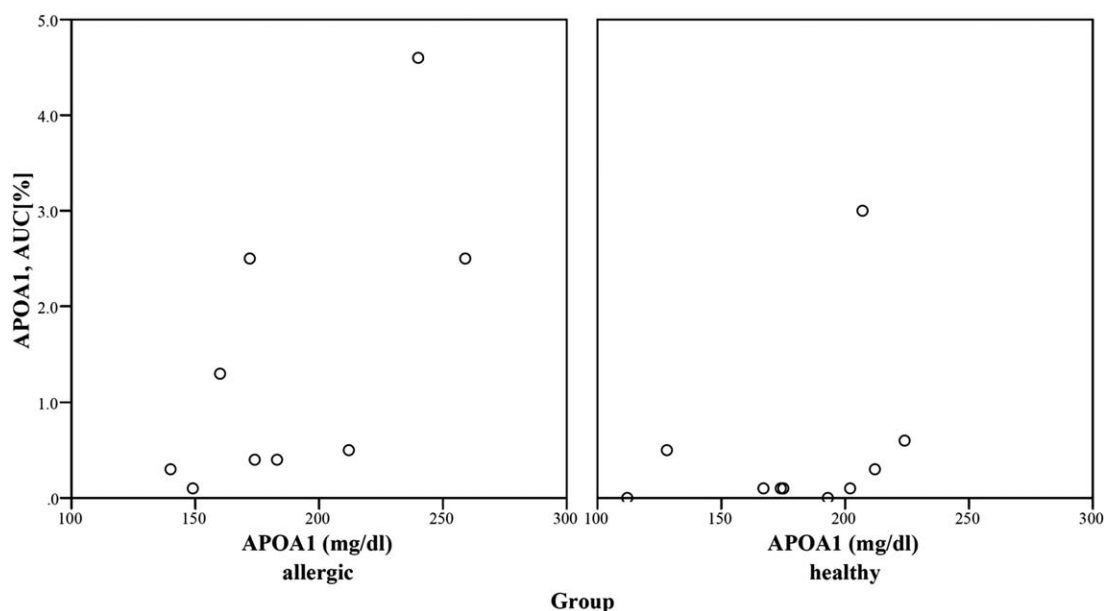


Fig. 3. Scatter diagram of correlation between mucus abundances (y-axis) and plasma concentrations (x-axis) of ApoA-I in allergic rhinitis patients and healthy controls..

and healthy controls are supposed to be greatest. We also applied a more sensitive and accurate method to quantify protein abundances by normalized areas under the curve instead of spectral counts, where a cutoff of four spectral counts or more is recommended. Thus, valuable quantitative information on low abundance proteins could be lost.²⁰

Interestingly, the removal of lipids from nasal fluid leads to decreased antimicrobial activity, particularly against *P. aeruginosa*, which can be restored after lipid supplementation.¹⁹ Beck et al.²¹ also showed antimicrobial activity of ApoA-I through binding to lipopolysaccharide (LPS), impairing the growth of *Klebsiella pneumoniae* and *Escherichia coli*.

Apart from their antimicrobial properties, apolipoproteins, particularly A-I and A-II, exert antiinflammatory properties. In isolated neutrophils, Furlaneto et al.²² discovered that upon stimulation with LPS, ApoA-I reduced IL-1 β release and ApoA-II reduced IL-8 release by 47% and 46%, respectively. Reactive oxygen species produced by neutrophils were reduced as well. In an ovalbumin-induced asthma mouse model, Dai et al.¹¹ showed that in APOA-I-depleted mice, granulocyte inflammation of alveolar tissue was significantly higher than in wild type mice and proposed a granulocyte colony stimulating factor mediated pathway. Additionally, proinflammatory cytokines, chemokines, and vascular cell adhesion molecules were up-regulated in ApoA-I depleted mice upon ovalbumin challenge. Park et al.¹⁰ showed that ApoA-I promotes recovery of tight junctions in bronchial epithelium of asthmatic patients by induction of lipoxin A4, which is important for the production of the tight junction proteins zonula occludens-1 and occludin. Taken together, apolipoproteins show antimicrobial and antiinflammatory properties and promote epithelial barrier recovery. The high abundance of apoli-

poproteins in allergic nasal mucus could counteract the degradation of nasal epithelial tight junctions through pollen proteases.²³ Since plasma levels of ApoA-I and mucus abundances did not correlate in allergic rhinitis patients—despite equal mucus volumes and physiologic plasma levels—a local mechanism of apolipoprotein release into nasal mucus is assumed. This could be triggered by allergen challenge as a subsequent antiinflammatory response and not by unspecific plasma exudation.⁷ The shortcoming of this study, however, is that we did not measure epithelial permeability for apolipoproteins, which could confirm a local mechanism.

Apart from an antiinflammatory role of apolipoproteins, a proinflammatory component is also proposed. The reason could be a posttranslational modification of apolipoproteins by myeloperoxidase, causing dysfunctional HDL particles and/or carbamylation of apolipoproteins.⁸ In our present study, myeloperoxidase was only found in three out of 10 allergic rhinitis patients compared to six out of 12 healthy controls. Furthermore, protein abundances were low and did not reach significant difference between the two groups. This finding weakens the hypothesis of a proinflammatory activity of apolipoproteins in nasal mucus. Moreover, the majority of studies underline an antiinflammatory function of apolipoproteins in allergy and asthma.⁸

CONCLUSION

Our newest findings confirm the significantly higher abundance of apolipoproteins A-I and A-II in allergic rhinitis mucus compared to healthy controls, thus supporting the important role of these proteins in allergic rhinitis. We suppose that their release is triggered by a local mechanism and not via plasma exudation because apolipoprotein A-I levels in mucus did not

correlate with its levels in plasma; this would need to be confirmed by measuring epithelial permeability for apolipoproteins. Apolipoproteins could be a response to allergen challenge and act as antiinflammatory and epithelium-protective molecules.

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