

Nasal Epithelium Biomarkers in Young Children: Associations With Allergic Sensitization and Environmental Stressors

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Summary. Background: Recent studies suggest that allergic diseases might have their onset in early epithelial barrier defects. To test this hypothesis, we assessed associations between nasal epithelium biomarkers, environmental stressors, and the risks of allergic sensitization. Methods: In a cross-sectional study of 288 kindergarten children (mean age, 5.7 years), we measured aeroallergen-specific IgE in nasal mucosa and the concentrations of urea, albumin, and Clara cell protein (CC16) in nasal lavage fluid (NALF). Albumin and CC16 in NALF were expressed per liter or as a ratio to urea. We also calculated the NALF CC16/albumin ratio as an index integrating the permeability and the secretory function of the nasal epithelium. Results: Median NALF concentrations of CC16 and albumin were 8.2 μ g/L and 34.5 mg/L, respectively. While there were no significant gender differences when proteins were expressed per liter, the CC16 to albumin and CC16 to urea ratios in NALF were higher in girls than in boys ($P = 0.02$ and 0.055 , respectively). The nasal epithelial barrier function, as reflected by these NALF biomarkers, was positively influenced by probiotics and age, and negatively by environmental stressors such as pool chlorine. The risk of house dust mite (HDM) sensitization increased with decreasing log NALF CC16 concentration, whether expressed per liter (2.59, 95% CI: 1.15–5.82, $P = 0.02$), as a ratio to urea (1.98, 95% CI: 0.96–4.06, $P = 0.06$), or as a ratio to albumin (OR, 2.03, 95% CI: 1.10–3.74, $P = 0.02$). Children in the highest and intermediate tertiles of the NALF albumin/urea ratio were three times more likely to be sensitized to HDM than those in the lowest tertile (both $P = 0.04$). Conclusion: Defects in the nasal epithelium barrier function of young children, as reflected by the concentrations of CC16 and albumin in NALF, are associated with environmental factors, including pool chlorine, and with increased risks of HDM sensitization. **Pediatr Pulmonol.** © 2012 Wiley Periodicals, Inc.

Key words: nasal epithelium; environmental tobacco smoke; Clara cell protein; albumin; chlorine; allergy; permeability; biomarkers.

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INTRODUCTION

Allergic diseases and asthma affect up to 30–40% of the population worldwide, and the prevalence of these diseases is likely to further increase in the future. Current hypotheses attribute this rise of allergies to the hygiene practices of Westernized societies. According to the classical hygiene hypothesis formulated in 1989, the increase of allergic diseases in developed countries would be the consequence of the reduced exposure to infective agents during early life.^{1,2} In 2003, however, a variant of the hygiene hypothesis was proposed that links the rise of allergies to the increasing exposure of children to products of chlorination, the most widely used method to achieve hygiene in the developed world.³ Basically, this hypothesis, called the chlorine hypothesis, postulates that chlorine-based oxidants used

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or released during water chlorination, in particular in swimming pools, cause changes in epithelial barriers that promote allergic sensitization and the development of allergic diseases.^{3,4}

The hypothesis linking the rise of allergies to epithelial barrier defects caused by environmental insults is consistent with the emerging concept that allergic diseases are primarily epithelial disorders.^{5,6} The structural or functional alterations in epithelial barriers likely to promote allergic sensitization are yet to be elucidated. A first possible mechanism is an epithelial hyperpermeability that would facilitate the transepithelial delivery of allergens to dendritic cells. This mechanism might be triggered by stressors capable of breaching tight junctions such as allergens with a proteolytic activity^{7,8} or chemicals with a strong oxidizing potential like ozone⁹ and chlorine-based oxidants.^{3,10} A second possible mechanism would be the destruction or the dedifferentiation of epithelial cells secreting immunomodulatory proteins. This mechanism might for instance involve Clara cell protein (CC16), a secretory protein of the airways that increasingly appears to play an important immunomodulatory role by downregulating the Th2 response.^{11–13} Decreased levels of CC16 have been reported in patients with allergic rhinitis and asthma^{14–16} and in subjects exposed to chlorination products and other environmental stressors.^{17,18}

Because allergic diseases have usually their onset in early life, the question of whether these diseases primarily arise from defective epithelial barriers can be explored only in very young children. Tests based on CC16 and other lung secretory proteins in serum require a blood sampling, which for ethical and practical reasons is not feasible in most epidemiological studies involving young children. An alternative approach would be to use epithelial biomarkers measurable in nasal lavage fluid (NALF), a biological material that can be collected in a totally noninvasive manner. According to “united airways concept,”^{19,20} there is indeed a close connection between upper and lower airways during the allergic response, suggesting that alterations found in nasal epithelium might mirror similar changes occurring in the deep lung.

In the search for noninvasive biomarkers applicable in very young children, we have recently explored the potential of albumin and CC16 in NALF to detect nasal epithelium changes related to environmental stressors and to the risks of IgE sensitization. In a study among school adolescents, we recently found that the CC16/albumin ratio in NALF, an index reflecting the permeability and secretory function of the nasal epithelium, was inversely associated with the risk of sensitization to house dust mite (HDM) and with various stressors, including irritants in chlorinated pools.²¹ The objective of the present study was to determine whether the assay of CC16 and albumin in NALF could be sufficiently sensitive to detect in young children variations in nasal epithelium function, which would be associated with IgE sensitization and with some environmental or host-related factors.

MATERIALS AND METHODS

Population

Study participants were 5–6 years old children from 30 kindergarten schools located in the Southern part of Belgium. These children were recruited in the framework of a cross-sectional study on the respiratory impact of air pollution as described elsewhere.²² They were examined only with the written informed consent of parents. In addition, just before performing the tests in schools, we verbally assured to have the assent of each child. Briefly, the protocol included a parent self-administered questionnaire concerning the health of children and their exposure to risk factors for allergic diseases. The questionnaire also addressed aspects related to social and medical characteristics of the children and their family, the in- and out-house environment and the attendance at chlorinated pools, both indoors and outdoors. Questions about respiratory symptoms and allergic diseases were taken from the International Study of Asthma and Allergy in Children. The initial population included 431 children of whom 288 could perform successfully the Rhinostick test and the collection of NALF samples from both nostrils. The Ethics Committee of the Faculty of Medicine of the Catholic University of Louvain approved the study protocol, which complied with all applicable requirements of international regulations.

Samples Collection and Analyses

Children were examined between December 11, 2007 and March 3, 2008. The examination, which took place in the schools between 9:00 and 16:00, included the measurement of height and body weight, a screening of aeroallergens-specific IgE in the nasal mucosa using the Rhinostick test and the collection of a NALF fluid from

ABBREVIATIONS:

NALF	Nasal lavage fluid
CC16	Clara cell protein
CPA	Cumulative pool attendance
ELF	Epithelial lining fluid
HDM	House-dust mite
IgE	Immunoglobulin E
IQR	Interquartile range
SD	Standard deviation

both nostrils as described previously.²¹ CC16 was quantified by a semi-automated nonisotopic immunoassay based on the agglutination of latex particles coated with a polyclonal antibody.^{23,24} We used a rabbit anti-human CC16 polyclonal antibody (A0257 purchased from DAKO: Glostrup, Denmark) and as standards the protein purified in our laboratory. The immunoassay was validated by comparison with a fluorescence enzyme immunoassay (FEIA) using monoclonal antibodies.²⁵ Albumin and urea were quantified by the Beckman Synchron CX5 Delta Clinical System. As reported previously, coefficients of variation (CVs) of CC16 and albumin determination in NALF samples are in the range of 30–40%, which is quite acceptable considering that these CVs integrate the analytical variation of immunoassays, the variable recovery of proteins from the nasal epithelial lining fluid (ELF) and the between-day biological variations in the ELF composition. We used the mean concentrations of albumin and CC16 determined in the NALF collected in each nostril separately. Concentrations were expressed per liter or as a ratio to the urea concentration in order to correct for the variable dilution of ELF during the nasal lavage.^{26,27} We adopted this procedure because as shown previously,²¹ there may be significant differences in the NALF concentrations of albumin and CC16 between the two nostrils, even after adjustment for the urea concentration. Under these conditions, the mean of the concentrations determined in each nostril separately provides a more reliable assessment of nasal epithelial function than a single measurement obtained after pooling NALF samples, the volume of which are variable depending on the recovery of instilled physiological saline. We also calculated the mean value of the CC16/albumin ratio in the NALF from both nostrils as an index integrating the opposite variations of albumin and CC16 in NALF that may occur when both the permeability and the secretory function of the nasal epithelium are impaired.

Statistical Analyses

All continuous variables were log-transformed before statistical analyses at the exception of age, the cumulative attendance at pools or day care center, the duration of breastfeeding, and the consumption of probiotics. Differences between boys or girls were compared by the Chi-squared test, the Mann–Whitney *U*-test or the Student's *t*-test depending on the type of variable. Continuous variables were reported as mean with SD or as median with interquartile range (IQR). Determinants of biomarkers concentrations in NALF were traced by multiple linear regression analysis (stepwise backwards procedure). We tested 27 potential predictors, including among others, age, birth month, day care attendance, breastfeeding, probiotics, pacifier use, older siblings, parental asthma or allergies,

parental smoking, mother smoking during pregnancy, pets, proximity of a busy road, chlorinated pool attendance (all types of pools combined), doctor-diagnosed allergic or respiratory diseases, and medication for allergies or asthma. We used a lifetime cumulative pool attendance (CPA) of 100 hr to categorize the subjects as having regularly attended a chlorinated pool. We used backward logistic regression models to calculate the odds ratios (ORs) of being sensitized against HDM or pollen according to the levels of CC16, albumin and of the CC16/albumin ratio in NALF. Biomarkers levels in NALF were stratified in tertiles of increasing (albumin) or decreasing (CC16 and the CC16/albumin ratio) values. The logistic regression models include all potential predictors mentioned above, with the exception of predictors that correlated with NALF biomarkers in multiple regression analyses. At each step, the least significant predictor was deleted until the model only contained variables with a *P*-value less than or equal to 0.20. All *P* values were two-sided and results were considered as statistically significant at *P* values below 0.05. All statistical analyses were performed using StatView (v5.0.1, 3rd ed., a business unit of SAS, 2001).

RESULTS

Characteristics of girls and boys are described in Table 1. The only noticeable differences between the two sexes concerned the use of a pacifier and the exposure to parental smoking, which were more frequent in boys than in girls. Otherwise, most risk factors of allergic diseases, including parental history of allergies, breastfeeding, exposure to pets or to older siblings, probiotics consumption, attendance of day care or of chlorinated swimming pools did not differ meaningfully between the two sexes. There were also no significant gender differences in the proportions of children sensitized to any aeroallergen (about 25% in both sexes) or of children suffering from allergic diseases and asthma, even though boys tended to be more frequently sensitized to HDM or to suffer from allergic rhinitis.

Table 2 compares NALF biomarker levels between boys and girls. The NALF concentration of urea was higher in boys than in girls (+18%, *P* = 0.02). There was no statistically significant gender difference in the levels of albumin whether expressed per liter (*P* = 0.07) or adjusted from the NALF urea concentration (*P* = 0.37). When expressed per liter, the NALF concentration of CC16 also did not differ between the two sexes (*P* = 0.22). By contrast, the NALF concentration of CC16 adjusted for the urea or albumin concentration in NALF were lower in boys than in girls (−22%, *P* = 0.055 and −35%, *P* = 0.03, respectively).

TABLE 1— Characteristics of Children

	Girls (N = 136)	Boys (N = 152)	P-value
Age, mean (SD), years	5.71 (0.35)	5.70 (0.33)	0.66
BMI, mean (SD), kg/cm ²	20.5 (3.83)	20.9 (2.64)	0.14
Birth weight, mean (SD), g	3,190 (677)	3,310 (627)	0.18
Birth between May and August, N (%)	38 (27.9)	56 (36.8)	0.11
Parental allergy, N (%)	56 (41.2)	70 (46.1)	0.41
Parental asthma, N (%)	19 (14.0)	16 (10.5)	0.37
Breastfeeding			
N (%)	108 (79.4)	122 (80.3)	0.86
Mean (SD), months	4.66 (5.26)	5.00 (5.02)	0.33
Day care attendance			
N (%)	77 (56.6)	59 (38.8)	0.15
Mean (SD), months	13.4 (13.0)	11.4 (12.8)	0.13
Pacifier use, N (%)	78 (57.4)	104 (68.4)	0.052
Smoking during pregnancy, N (%)	23 (16.9)	15 (9.87)	0.08
Parental smoking at home, N (%)	47 (34.6)	35 (23.0)	0.03
Number of older siblings, mean (SD)	0.82 (1.02)	0.89 (0.96)	0.34
House cleaning with bleach, N (%)	33 (24.3)	37 (24.3)	0.99
Exposure to pets since birth, N (%)	69 (50.7)	83 (54.6)	0.51
Living at <100 m from a busy road, N (%)	50 (36.7)	48 (31.6)	0.35
Living in Liège (industrial city) N (%)	50 (36.8)	66 (43.4)	0.25
Vaccination, N (%)	106 (77.9)	107 (70.4)	0.15
Indoor chlorinated pool attendance			
N (%)	122 (89.7)	138 (90.8)	0.76
Median (IQR), hours	41.4 (15.4–115)	53.8 (21.3–145)	0.17
Outdoor chlorinated pool attendance			
N (%)	81 (59.6)	86 (56.6)	0.61
Median (IQR), hours	18.0 (0.0–76)	17.3 (0.00–103)	0.26
Probiotics consumption for week,			
N (%)	53 (39.0)	70 (46.1)	0.23
Mean (SD)	1.31 (2.31)	1.73 (3.10)	0.36
Sensitization to aeroallergens			
At least one aeroallergen, N (%)	33 (24.3)	39 (25.7)	0.79
House dust mite, N (%)	13 (9.6)	22 (14.5)	0.20
Cat epithelium, N (%)	14 (10.3)	11 (7.2)	0.36
Pollen, N (%)	19 (14.0)	24 (15.8)	0.67
Frequent colds, N (%)	57 (41.9)	68 (44.7)	0.63
Sinusitis, N (%)	20 (14.7)	20 (13.2)	0.70
Hay fever, N (%)	5 (3.7)	7 (4.6)	0.69
Allergic rhinitis, N (%)	8 (5.9)	18 (11.8)	0.08
Asthma, N (%)	9 (6.6)	13 (8.6)	0.54
Medication for allergic diseases, N (%)	22 (16.2)	35 (23.0)	0.15

TABLE 2— Biomarkers in the NALF of Boys and Girls

Parameters	Girls (N = 146)	Boys (N = 155)	P-value
Urea (mg/L)	49.0 (38.8–68.8)	58.0 (41.8–78.8)	0.02
Albumin (mg/L)	30.6 (15.3–57.0)	38.4 (17.6–70.1)	0.07
Albumin/urea ratio	0.57 (0.32–1.03)	0.63 (0.34–1.16)	0.37
CC16 (μg/L)	9.20 (4.18–20.0)	7.45 (3.83–14.90)	0.22
CC16/urea ratio ($\times 10^{-3}$)	0.18 (0.08–4.69)	0.14 (0.06–2.78)	0.055
CC16/albumin ratio ($\times 10^{-4}$)	3.25 (1.23–10.5)	2.10 (0.91–6.64)	0.03

Concentrations are expressed as median with interquartile range. The median NALF concentration for the whole population were: albumin, 34.5 mg/L; CC16, 8.2 μg/L.

Factors associated with NALF levels of CC16 and of albumin and their ratio in NALF were traced by multiple regression analyses testing the effects of 27 potential predictors. Two models were used to adjust the NALF concentrations of CC16 and albumin for the variable dilution of the ELF during the lavage procedure. In the first model, we expressed the protein concentrations per liter of NALF and we added the NALF concentration of urea in the list of independent variables. In the second model, we expressed the concentrations of NALF proteins as a ratio to urea concentration. Because of the gender differences in the levels of CC16 and of the CC16/albumin ratio, we performed these analyses separately for girls and boys. As shown in Table 3, the most prominent determinant of NALF biomarkers in girls was the probiotics consumption that was associated with a more efficient nasal epithelium barrier as reflected by an increase of CC16, a decrease of albumin, and a highly significant increase of the CC16/albumin ratio in NALF. In girls, the only consistent predictor of an impairment of nasal epithelium was the chlorinated pool attendance that was associated with a decrease of NALF CC16, expressed per liter or as a ratio to urea or to albumin. Sensitization to HDM was also associated with an impaired nasal epithelium function but the association emerged only through the CC16/albumin ratio. Altered levels of CC16 and albumin were seen in the NALF of girls who reported frequent cold but these associations did not persist after adjustment for urea. Of note, despite the narrow age

range of our participants, the NALF CC16/albumin ratio increased significantly with age in girls but also in boys. Actually this positive influence of age on the CC16/albumin ratio was the only significant association seen in both sexes. In boys, indeed, the NALF CC16/albumin ratio showed an inverse association with sensitization to pollen but not with sensitization to HDM. Associations were seen also with frequent cold but these concerned different biomarkers and were in the opposite direction to that seen in girls. Boys living in a house regularly cleaned with bleach had lower levels of CC16 and albumin in NALF. None of the NALF biomarkers measured in boys correlated with chlorinated pool attendance.

Associations between NALF biomarkers and allergic sensitization were further assessed in logistic regression analyses by testing sensitization to HDM or pollen not as a predictor of NALF biomarkers but as a possible outcome of altered epithelial changes detected by these biomarkers. Different models were built in which NALF biomarkers were tested as continuous variables or as tertiles of increasing (albumin) or decreasing (CC16 and the CC16/albumin ratio) values. Analyses were conducted by testing the same covariates as in stepwise regression analyses at the exception of covariates that were correlated significantly with NALF biomarkers in stepwise regressions. Because of the strong influence of gender on the levels and covariates of CC16 and CC16/albumin ratio in NALF, these analyses were done for both the whole population and for boys

TABLE 3—Factors Influencing the Concentrations of Urea, CC16 and Albumin NALF of Girls and Boys

Dependent variable	Girls (n = 146)			Boys (n = 155)		
	Independent variable	Regression coefficient (95% CI)	P-value	Independent variable	Regression coefficient (95% CI)	P-value
Log CC16 ($\mu\text{g/L}$)	Frequent colds	-0.24 (-0.40 to -0.07)	0.006	Age	0.27 (0.03–0.50)	0.02
	Parental smoking	0.24 (0.07 to 0.41)	0.007	Bleach	-0.21 (-0.39 to -0.03)	0.03
Log CC16/urea ratio	CPA > 100 hr	-0.18 (-0.35 to -0.02)	0.03	Age	0.34 (0.09–0.58)	0.008
	CPA > 100 hr	-0.22 (-0.40 to -0.05)	0.01	Frequent colds	-0.17 (-0.33 to -0.01)	0.043
	Probiotics	0.04 (0.01–0.08)	0.02	Bleach	-0.26 (-0.45 to -0.07)	0.008
	Parental smoking	0.19 (0.01–0.38)	0.04			
Log Albumin (mg/L)	Living in Liège	0.19 (-0.01 to 0.38)	0.04	Frequent cold	0.13 (0.004–0.27)	0.044
	Probiotics	-0.04 (-0.07 to -0.01)	0.007			
	HDM IgE	0.24 (0.0004–0.47)	0.049	Living in Liège	0.17 (0.04–0.30)	0.01
Log Albumin/urea ratio	Frequent colds	-0.17 (-0.31 to -0.03)	0.02	Pollen IgE	0.23 (0.05–0.40)	0.01
	Probiotics	-0.04 (-0.06 to -0.01)	0.01	Bleach	-0.22 (-0.37 to -0.07)	0.004
Log CC16/albumin ratio	Probiotics	0.08 (0.03–0.12)	0.002	Frequent colds	-0.27 (-0.47 to -0.08)	0.007
	HDM IgE	-0.52 (-0.88 to -0.17)	0.005	Pollen IgE	-0.37 (-0.64 to -0.10)	0.008
	CPA > 100 hr	-0.22 (-0.49 to -0.01)	0.04	Age	0.31 (0.01–0.61)	0.044
	Age	0.38 (0.08–0.69)	0.02			

CPA, cumulative pool attendance; HDM, house dust mite; CC16, Clara cell protein.

and girls, separately. We found no significant associations between any NALF biomarker and the risk of sensitization to pollen. By contrast, the risk of sensitization to HDM increased with decreasing log of urea-adjusted CC16 (1.98, 95% CI, 0.96–4.06, $P = 0.06$) and log of CC16/albumin ratio in NALF (OR, 2.03, 95% CI 1.10–3.74, $P = 0.02$). Depending on the tested model, odds for HDM sensitization associated with NALF biomarkers were higher in girls and in boys but overall there was no systematic gender difference, meaning that these associations are not driven by one sex only (Table 4).

DISCUSSION

The aim of the present study was to further explore the environment or lifestyle stressors that might affect nasal epithelial barriers and thereby increase the risks of allergic sensitization during childhood. The integrity of the nasal epithelium of children was assessed noninvasively by measuring two proteins in NALF. The first protein is albumin, a plasma-derived albumin that is a

well-established biomarker of epithelial permeability in respiratory tract-derived fluids, including the NALF.^{28–30} The second protein is the 16 kDa Clara cell protein, an anti-inflammatory protein secreted in large amounts by the nasal epithelium,^{15,16} which increasingly appears to play a central role in the development of asthma and allergic diseases.^{11,12} We also used the ratio between CC16 and albumin in NALF as an index theoretically independent of the ELF dilution during the lavage procedure and reflecting both the increased permeability and the decreased secretory function of the nasal epithelium.

The results confirm the existence of the associations found previously²¹ between the sensitization to HDM and the integrity of the nasal epithelium assessed on the basis of CC16 and albumin in NALF. Because of the cross-sectional nature of our study, no firm conclusion can be drawn regarding the direction of causality in these associations. A first possibility would be that these associations arise as a consequence of the breaching of airway epithelial barriers by the proteolytic activity of allergens. It should be stressed, however, that

TABLE 4—Risks of Sensitization to House Dust Mite According to Increasing Levels of Albumin and Decreasing Levels of CC16 and of the CC16/Albumin Ratio in the NALF of all Children, or of Girls and Boys Separately

	All (OR 95% CI)	<i>P</i> -value	Girls (OR 95% CI)	<i>P</i> -value	Boys (OR 95% CI)	<i>P</i> -value
CC16 (μg/L)						
Continuous (log)	2.59 (1.15–5.82)	0.02	1.87 (0.53–6.53)	0.33	3.48 (1.11–11.0)	0.03
Tertile 1 (highest)	1.00 (1.00–1.00)		1.00 (1.00–1.00)		1.00 (1.00–1.00)	
Tertile 2	2.80 (1.03–6.95)	0.04	4.03 (0.87–18.6)	0.07	1.69 (0.63–13.39)	0.42
Tertile 3 (lowest)	2.37 (0.84–6.65)	0.10	1.82 (0.44–10.4)	0.45	1.93 (0.54–6.86)	0.31
<i>P</i> for trend	0.12		0.32		0.39	
CC16/urea ratio						
Continuous (log)	1.98 (0.96–4.06)	0.06	2.39 (0.74–7.76)	0.15	1.65 (0.62–4.42)	0.33
Tertile 1 (highest)	1.00 (1.00–1.00)		1.00 (1.00–1.00)		1.00 (1.00–1.00)	
Tertile 2	2.65 (1.00–7.04)	0.051	2.10 (0.50–8.89)	0.31	1.00 (0.30–3.33)	0.96
Tertile 3 (lowest)	2.33 (0.86–6.33)	0.10	1.91 (0.45–8.14)	0.38	1.27 (0.38–4.27)	0.70
<i>P</i> for trend	0.19		0.32		0.97	
Albumin (mg/L)						
Continuous (log)	1.75 (0.72–4.24)	0.21	2.40 (0.57–10.2)	0.23	0.88 (0.27–2.54)	0.86
Tertile 1 (lowest)	1.00 (1.00–1.00)		1.00 (1.00–1.00)		1.00 (1.00–1.00)	
Tertile 2	2.26 (0.91–5.60)	0.08	3.70 (0.67–20.5)	0.36	1.62 (0.52–5.09)	0.41
Tertile 3 (highest)	1.21 (0.45–3.26)	0.41	2.77 (0.28–15.8)	0.26	0.82 (0.22–2.99)	0.76
<i>P</i> for trend	0.66		0.16		0.71	
Albumin/urea ratio						
Continuous	1.97 (0.77–5.02)	0.16	1.51 (0.32–7.10)	0.60	2.37 (0.70–7.98)	0.17
Tertile 1 (lowest)	1.00 (1.00–1.00)		1.00 (1.00–1.00)		1.00 (1.00–1.00)	
Tertile 2	3.16 (1.08–9.22)	0.04	3.47 (0.62–19.3)	0.16	4.91 (1.14–3.13)	0.96
Tertile 3 (highest)	3.15 (1.07–9.22)	0.04	2.57 (0.45–14.6)	0.29	3.84 (0.88–16.9)	0.07
<i>P</i> for trend	0.03		0.16		0.16	
CC16/albumin ratio						
Continuous (log)	2.03 (1.10–3.74)	0.02	2.16 (0.86–5.43)	0.10	1.87 (0.79–4.41)	0.16
Tertile 1 (highest)	1.00 (1.00–1.00)		1.00 (1.00–1.00)		1.00 (1.00–1.00)	
Tertile 2	1.06 (0.39–2.86)	0.92	1.90 (0.39–9.35)	0.43	0.70 (0.17–2.92)	0.96
Tertile 3 (lowest)	2.27 (0.91–5.63)	0.08	3.18 (0.71–14.3)	0.13	1.27 (0.38–4.27)	0.70
<i>P</i> for trend	0.08		0.09		0.97	

CC16, Clara cell protein.

there is so far no evidence that *in vivo* proteolytic allergens are capable to cleave tight junctions of airway epithelium. A recent study found no change in airways epithelial barrier function in mice exposed by inhalation to HDM.³¹ A second possibility would be a decrease of CC16 secretion by the nasal epithelium consecutive to a dedifferentiation of Clara cells into goblet and/of ciliated cells occurring in allergic rhinitis.³² This possibility might account for the decrease of CC16 but not for the increase of albumin in the NALF of our children. A third possibility, which might be the most likely explanation, would be that NALF biomarkers reflect functional or structural alterations of epithelial barrier promoting allergic sensitization. Whether innate or acquired, these alterations might lead to an epithelial hyperpermeability enhancing the transepithelial delivery of allergens as well as the transepithelial loss of immunomodulatory proteins such as CC16. These defects might also consist in a dysfunction or depletion of Clara cells leading to a diminution of their immunoregulatory activity in airways. The fact that NALF biomarkers are more associated with HDM than with pollen sensitization might be explained by differences in the proteolytic activity of the allergens or, which is more probable, by differences in the exposure patterns of pollen and HDM allergens. To promote allergic sensitization, epithelial barrier defects require indeed a concomitant and sufficient exposure to allergens, two conditions that are more easily met with perennial allergens such as HDM than with seasonal allergens linked to pollination.

The hypothesis linking allergic sensitization to epithelial barrier disruption caused by environmental stressors is also supported by the determinants of NALF biomarkers that were already emerging in our children. Quite interestingly, the few determinants that were identified were all, in one way or another, linked to hygiene. Of these, there is the cumulative attendance at chlorinated pools, which was associated with a decrease of CC16 and of the CC16/albumin ratio in the NALF of girls. The only factor that appeared to be beneficial for the nasal barrier function was the consumption of probiotics, which in girls was associated with higher CC16 and lower albumin levels in the NALF. This finding is in accordance with a recent study suggesting that probiotics contribute to improve epithelial barrier function.³³ There is also an apparent protective effect of house cleaning with bleach on the permeability of nasal epithelium in boys, an association reminiscent of the protective effect of bleach towards the sensitization to indoor allergens.³⁴ As observed previously,²¹ factors associated with NALF biomarkers were not the same in boys and girls, age being the only common predictor seen in the two sexes.

To our knowledge, our study is the first to have measured CC16 and albumin in the NALF of very young children. Concentrations of urea in the NALF of children (median, 53.8 mg/L) were slightly higher than those found in the NALF of adolescents²¹ (median, 41.3 mg/L). This means that despite the very young age of our participants, the recovery of ELF was if anything slightly better than that in adolescents. Interestingly, the concentrations of NALF proteins—which were measured by exactly the same methods—were noticeably different between children and adolescents. By comparison with adolescents,²¹ the median NALF concentration of CC16 in our children was almost three times lower (8.2 μ g/L vs. 21.3 μ g/L) while that of albumin, on the contrary, was four times higher (34.5 vs. 8.5 mg/L). As a consequence, the CC16/albumin ratio integrating these opposite differences was almost 10 times lower in children than in adolescents (3.25 vs. 29.5×10^{-4}). These observations suggest that till puberty the nasal epithelium of young children undergoes an important maturation characterized by a progressive decrease of the epithelial permeability and an increase of the CC16 secretion. If the NALF CC16/albumin ratio reflects epithelial barriers defects predisposing to allergic sensitization, the fact that this ratio is so low during early childhood might explain why allergic sensitization has often its onset during early life.

In conclusion, sensitization to HDM during early childhood is associated with nasal epithelial defects as revealed by an increase of albumin and a decrease of CC16 concentrations in NALF. This association might reflect an increased risk of sensitization as a consequence of an acquired or innate epithelial barriers disruption, even if the possibility of a reverse causation due to the HDM allergen's own proteolytic activity cannot be excluded. Our study also provides evidence that the nasal epithelial barrier function can be modulated very early in life, positively by the consumption of probiotics and negatively by environmental stressors like pool chlorine.

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