



Nasal epithelium injury by chlorination products and other stressors predicts persistent sensitization to aeroallergens in young schoolchildren



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ABSTRACT

Background: Allergic sensitization during childhood is a dynamic process with a substantial rate of remission. Factors influencing this process are largely unknown.

Methods: We conducted a two-year prospective study among 121 schoolchildren (mean age, 5.8 years; 64 boys). We measured urea, club cell protein (CC16), β_2 -microglobulin and albumin in nasal lavage fluid (NALF) and IgE to cat, pollen or house dust mite (HDM) in nasal mucosa fluid.

Results: Odds of persistent sensitization to any aeroallergen increased across baseline ascending tertiles of urea-adjusted β_2 -microglobulin or albumin and descending tertiles of albumin- or β_2 -microglobulin-adjusted CC16 (P -trend = 0.006, 0.02, 0.044 and 0.006, respectively). Persistent HDM sensitization also increased with baseline descending tertiles of raw or urea-adjusted CC16 (both P -trend = 0.007). Such strong associations were not observed with new-onset or remitted sensitization to any aeroallergen or with raw NALF concentrations of urea, albumin or β_2 -microglobulin. At baseline, house cleaning with bleach and chlorinated pool attendance emerged among the strongest and most consistent determinants of NALF biomarkers, being both associated with higher urea and lower CC16 in NALF.

Conclusion: In young children, a defective nasal epithelium attributable to immaturity or stressors such as chlorination products is predictive of more persistent aeroallergen sensitization.

1. Introduction

Since the 1970s, the prevalence of allergic diseases such as asthma and hay fever has dramatically increased in affluent societies, affecting up to one third of the population (Muraro et al., 2016). While considerable advances have been made in understanding the mechanisms of allergies, much less is known about factors driving the epidemic of allergies. Initially, it was hypothesized that allergies were the consequences of Western hygiene practices, which by reducing microbial exposure during early life would shift the immune system towards a Th2 dominant response (Strachan, 1989). A more recent hypothesis proposes that the rise of allergies is primarily due to epithelial barrier defects caused by changes in lifestyle or environment (Holgate, 2011). There is growing evidence indeed that airway epithelium alterations play a key role in the development of allergic diseases, contributing to both the allergic sensitization and its clinical expression (Lambrecht and Hammad, 2012). Studies of patients with asthma or allergic rhinitis have revealed a number of structural or functional defects of the airway epithelium, including increased tight junction permeability, airway

wall remodelling, club cell loss and goblet cell metaplasia (Holgate et al., 2000; Fedorov et al., 2005; Kim et al., 2010; Steelant et al., 2016). According to the epithelial hypothesis, some of these defects could promote allergic diseases by facilitating the transepithelial delivery of allergens to dendritic cells and/or by decreasing the secretion of mediators such as the club cell protein (CC16) down-regulating the Th2 differentiation and the inflammatory response (Hung et al., 2004; Johansson et al., 2007; Tokita et al., 2014).

The major challenge for epidemiological studies testing the epithelial hypothesis is to assess non-invasively the airway epithelium integrity. One possible approach consists of measuring the serum concentrations of lung-specific proteins (also named pneumoproteins), which are biomarkers reflecting the structural or functional integrity of the pulmonary epithelium (Hermans and Bernard, 1998, 1999). When for ethical reasons it is not possible to collect blood (e.g. in schoolchildren), an alternative approach is to use epithelial biomarkers measurable in nasal lavage fluid (NALF) (Sardella et al., 2012, 2013). Studies using these peripheral airway biomarkers have shown that various stressors including air pollutants, strenuous exercise or

Abbreviations: Alb, albumin; β_2 -m, β_2 -microglobulin; CC16, club cell protein; CPs, chlorination products; CPA, cumulative pool attendance; ELF, epithelial lining fluid; eNO, exhaled nitric oxide; HDM, house dust mite; IQR, interquartile range; NALF, nasal lavage fluid; OR, odds ratio

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endotoxin can cause acute or chronic damage to the airway epithelial barriers (Broekaert and Bernard, 2000; Lagerkvist et al., 2004; Nanson et al., 2001; Michel et al., 2005). Among these stressors, chlorination products (CPs) are of particular interest because these strong oxidants linked to the hygiene of the developed world are well-known for their capacity to breach tight junctions (Bernard, 2007). In addition, epidemiological evidence suggests that exposure to CPs in swimming pools, especially during early life, may increase the risk of allergic sensitization or diseases (Bernard et al., 2003, 2008; Bougault et al., 2012; Voisin et al., 2014; Andersson et al., 2015). Supporting these findings, recent biomarker studies among school adolescents or children have demonstrated that the disruption of airway epithelium by CPs correlated with the risk of allergic sensitization, especially to house dust mite (HDM), and among sensitized subjects, with the risk of developing asthma or hay fever (Sardella et al., 2012, 2013; Bernard et al., 2015a, 2015b).

However, because of their cross-sectional design, biomarker studies conducted until now cannot be used to infer a causal relationship between allergic sensitization and loss of epithelial barrier function associated with environmental stressors. The reason is that the impaired barrier function can be also the consequence of the sustained Th2-mediated inflammation and/or of the proteolytic activity of aeroallergens from pollen, fungi or HDM (Vinh et al., 2011; Leino et al., 2013; Wan et al., 1999). In an attempt to resolve these issues, we prospectively investigated in young children the associations between nasal epithelium defects and the risks of new-onset or persistent sensitization to most common aeroallergens. We evaluated the integrity of the nasal epithelium by measuring in nasal lavage fluid (NALF) CC16 as a marker of cell damage/dysfunction and plasma-derived albumin and β_2 -microglobulin (β_2 -m) as markers of epithelial permeability.

2. Materials and methods

Study participants were children (53% boys, mean age at baseline 5.8 years) who were recruited and examined in 30 schools in Belgium. As described in detail elsewhere (Voisin et al., 2010), a total of 430 children participated in the baseline examination. Two years later, among the 310 children still in schools, 236 (78.1%) participated to the examination and of them 121 could successfully perform all the tests, both at baseline and at follow-up. The ethics committee of the faculty of medicine of the catholic university of Louvain approved the study protocol. Children were examined with the signed informed consent of parents and their oral assent at time of examination.

2.1. Questionnaire

We used a parent self-administered questionnaire to collect data about the child's medical history, environment and lifestyle. For swimming practice, the questionnaire included specific questions to calculate the cumulative hours the child had spent in indoor and/or outdoor chlorinated pools (CPA, cumulative pool attendance), over lifetime and over the two years preceding baseline examination. Parents were also asked whether their child had ever been diagnosed as having asthma, hay fever or perennial allergic rhinitis.

2.2. Samples collection and analyses

We examined children in their schools between 9:00 A.M. and 3:00 P.M. The protocol comprised the measurement of height, body weight and exhaled nitric oxide (eNO, NIOX analyser, Aerocrine AB, Solna, Sweden) and a screening of specific IgE to cat dander, house-dust mite and grass (*anthoxanthum odoratum*), weeds (*Parietaria officinalis*) or tree (*betula odorosa*, *Corylus avellana*, *Carpinus betulus*, *Alnus incana*) pollen. These specific IgE were measured in nasal mucosa fluid using the Rhinostick test, a non-invasive method with a similar sensitivity but a greater specificity than skin-prick tests (Marcucci et al., 2004). The

Rhinostick test was calibrated with serum standards and considered positive at specific IgE ≥ 0.35 kIU/L. As described elsewhere (Sardella et al., 2012), NALF was collected by instilling two and a half ml of sterile physiological water at 37 °C into each nostril. The concentrations of CC16 and β_2 -m were measured by non-isotopic immunoassays based on the agglutination of latex particles (Bernard et al., 1992; Hermans et al., 1998). Albumin and urea were quantified by the Beckman Synchron CX5 Delta Clinical System. Concentrations of biomarkers in NALF were expressed as raw concentrations or as concentrations adjusted to the median NALF concentration of urea (i.e. as the ratio to urea concentration multiplied by the median urea concentration at baseline or follow-up) in order to correct for variations in the epithelial lining fluid (ELF) recovery (Cavaliere et al., 1986). We similarly adjusted the NALF concentration of CC16 to the median NALF concentrations of albumin or β_2 -m as indices integrating the decrease/dysfunction of CC16-secreting cells with the increased epithelial permeability. The use of β_2 -m in addition to albumin as permeability biomarker presents theoretically two advantages. First, the adjustment with β_2 -m, a plasma protein with a size close to that of CC16 (12 vs 16 kDa) should allow a more precise adjustment of CC16 levels in NALF for the transepithelial leakage of CC16 from plasma into the ELF. Second, because of its small size, β_2 -m could detect slight enlargements of the paracellular pores that would pass unseen with albumin. All these biomarkers were determined in NALF collected from each nostril separately and the mean value was calculated for the statistical analyses.

2.3. Statistical analyses

Concentrations of biomarkers were reported as median with inter-quartile range (IQR) and were log-transformed to approximate normal distribution. Differences between baseline and follow-up values were assessed using the chi-squared test or the paired Student's *t*-test. We used Pearson's correlation analysis to evaluate the univariate associations between the different NALF biomarkers at baseline or follow-up as well as between levels of the same biomarker at baseline and at follow-up. Associations of NALF biomarkers with the risks of new-onset, remitted or persistent aeroallergen sensitization were assessed by logistic regression models in which children were categorized in tertiles of descending CC16 or ascending urea, β_2 -m and albumin in NALF. We run these models by testing the raw or adjusted concentrations of NALF biomarkers and by considering various possible other predictors including gender, parental allergy or asthma, parental smoking, breastfeeding, day care attendance, chlorinated pool attendance or house cleaning with bleach. Although none of these other predictors was associated with the process of allergic sensitization, we decided to refine the odds ratios estimation by an a priori adjustment for gender and parental asthma/allergy, which are two well established predictors of allergic diseases. As there were less than 10% differences between crude and adjusted odds ratios, only the latter were reported in the Tables. Trends in odds across tertiles of NALF biomarkers were evaluated using the Cochran Armitage test. Factors influencing the baseline levels of NALF biomarkers were then traced by multiple regression analyses with variable selection based on the Akaike information criterion. We tested a total of 24 variables including age, gender, parental asthma or allergy, active or passive smoking, chlorinated pool attendance and a variety of other lifestyle- or environment-related factors (See Table S1 in Supplementary material for the complete list). Statistical analyses and graphs were made with the JMP 12.00 from SAS Institute. All *P*-values were two-sided with a level of statistical significance at *P* < 0.05.

3. Results

The cohort included 121 children (52.9% of boys) with a baseline mean age of 5.8 years. The characteristics of children are presented in Table 1 that combines both sexes as there were no significant gender

Table 1
Characteristics of children (n = 121).

Boys, N (%)	64 (52.9)
Age, years, median (IQR)	5.75 (5.50–5.92)
BMI, kg/m ² , median (IQR)	20.5 (18.4–22.1)
Mother with asthma and/or allergy, N (%)	28 (23.1)
Father with asthma and/or allergy, N (%)	22 (18.2)
Smoking during pregnancy, N (%)	15 (12.4)
Day care attendance, N (%)	68 (56.2)
Breastfeeding, N (%)	101 (83.5)
Older siblings, N (%)	71 (58.7)
Parental smoking, N (%)	26 (21.5)
Living < 100 from a busy road, N (%)	65 (53.7)
House cleaning with chlorine bleach, N (%)	23 (19.0)
Indoor and/or outdoor chlorinated pools	
Ever attendance, N (%)	112 (92.6)
Lifetime attendance, hours, median (IQR)	72 (24–143)
Doctor-diagnosed respiratory diseases, N (%)	
Asthma	13 (10.7)
Perennial allergic rhinitis	17 (14.1)
Hay fever	6 (5.0)

differences in studied outcomes. Compared with the 309 non-participants, participants were less exposed to parental smoking (21.5% vs 33.0%, $P = 0.02$) and they had been more frequently breastfed (83.5% vs 73.8%, $P = 0.03$). Otherwise, participants and non-participants did not differ significantly with respect to parental asthma or allergy (mother, 23.1% vs 29.8%, $P = 0.17$; father, 18.2% vs 25.9%, $P = 0.09$), older siblings (58.7% vs 54.4%, $P = 0.42$), day care attendance (56.2% vs 52.1%, $P = 0.44$), vicinity to a busy road (53.7% vs 61.2%, $P = 0.16$), use of bleach at home (19.0% vs 27.5%, $P = 0.07$), lifetime CPA (median hours, 85 vs 70, $P = 0.90$) and prevalence of ever-diagnosed asthma (10.7% vs 5.8%, $P = 0.08$), hay fever (5.0% vs 6.1%, $P = 0.64$) or of perennial allergic rhinitis (14.1% vs 11.3%, $P = 0.44$).

The raw NALF concentrations of urea, β_2 -m and albumin correlated with each other, both at baseline and at follow-up ($r = 0.23$ – 0.51 , $P < 0.01$, See Table S2 in Supplementary material). By contrast, the raw NALF concentration of CC16 showed no significant correlation with albumin or β_2 -m at baseline while at follow-up it correlated only with the raw or urea-adjusted concentration of β_2 -m ($r = 0.41$ and 0.34 , respectively, $P < 0.01$). Follow-up and baseline raw or adjusted concentrations of CC16 and albumin were significantly correlated whether considering all children or separately those with ($n = 29$) or without allergic diseases ($n = 92$) (Details in Table S3 in Supplementary material). Such persistent correlations during the follow-up were not found with the urea or β_2 -m concentrations in NALF.

Table 2 shows changes in prevalence of allergic sensitization and levels of NALF biomarkers and eNO during the follow-up. Compared to baseline, the prevalence of sensitization to HDM had increased (26.4% vs. 16.5%, $P = 0.049$) while the prevalence of sensitization to pollen or cat dander remained unchanged. At follow-up, the concentration of urea had increased by 25.0% ($P < 0.001$) while the urea-adjusted β_2 -m and the raw or urea-adjusted albumin concentrations had decreased by 20.0% ($P = 0.006$), 17.4% ($P = 0.03$) and 36.0% ($P < 0.001$), respectively. By contrast, there was a substantial increase, by about 300%, of the raw and urea-, β_2 -m- or albumin-adjusted concentrations of CC16 (all $P < 0.001$).

The individual follow-up of children confirmed the dynamic character of allergic sensitization at this young age. Considering sensitization to any aeroallergen, there were at the end of the follow-up 24 cases of remission (remission rate, 60%), 26 cases of new-onset and 16 cases of persistent sensitization. As depicted in Fig. 1 and in Fig. S1 of the supplementary material, all these cases had at follow-up similar levels of NALF biomarkers and eNO as their peers who were never-sensitized in the course of our study. At baseline, however, children with any aeroallergen sensitization differed from their never-sensitized peers as having significantly lower levels of albumin-adjusted CC16 and higher

Table 2
Biomarkers levels (n = 121) in nasal fluid and exhaled air at baseline and at follow-up.

	Baseline	Follow-up	P-value
Aeroallergen-specific nasal IgE, N (%)			
Any aeroallergen	40 (33.1)	42 (34.7)	0.78
House dust mite	20 (16.5)	32 (26.4)	0.046
Cat dander	15 (12.4)	11 (9.0)	0.41
Any pollen	25 (20.7)	17 (14.0)	0.13
Grass pollen	10 (8.3)	9 (7.4)	0.80
Tree pollen	11 (9.1)	9 (7.4)	0.59
Weed pollen	9 (7.4)	6 (5.0)	0.44
NALF biomarkers, median (IQR)			
Urea, mg/l	56.0 (43.3–84.3)	70.0 (54.5–94.0)	< 0.001
β_2 -m, μ g/l	834 (495–1390)	782 (341–1170)	0.67
β_2 -m adjusted for urea, μ g/l	812 (585–1220)	646 (417–947)	0.006
Albumin, mg/l	34.4 (17.2–66.6)	28.4 (13.3–56.0)	0.03
Albumin adjusted for urea, mg/l	32.8 (19.6–63.5)	21.0 (11.1–45.4)	< 0.001
CC16, μ g/l	7.6 (3.8–17.7)	30.8 (11.4–67.5)	< 0.001
CC16 adjusted for urea, μ g/l	6.6 (3.5–17.6)	24.3 (9.6–54.1)	< 0.001
CC16 adjusted for β_2 -m, μ g/l	8.8 (3.1–21.8)	31.5 (15.5–63.9)	< 0.001
CC16 adjusted for Alb, μ g/l	7.7 (2.9–18.5)	28.9 (10.3–79.8)	< 0.001
Exhaled NO, ppb, median (IQR)	7.5 (5.1–11.1)	11.0 (7.3–16.8)	< 0.001

Adjusted for the median values of urea, β_2 -m or albumin in the baseline or follow-up.

levels of eNO and of urea-adjusted β_2 -m and albumin. Fig. 1 also shows that changes in NALF biomarkers through the follow-up period did not differ according to the sensitization status (ANOVA on the log of the follow-up/baseline ratios, all $P > 0.05$). Levels of eNO, however, increased during follow-up in never sensitized children and in children with new-onset sensitization (Fig. S1 in Supplementary material).

We further assessed associations between nasal epithelium biomarkers and aeroallergen sensitization by calculating the odds of persistent, new-onset or remitted sensitization in children stratified in tertiles of baseline NALF levels of biomarkers while adjusting for gender and parental asthma or allergy. As shown in Table 3, the odds of persistent sensitization to any aeroallergen increased dose-dependently across ascending tertiles of urea-adjusted β_2 -m and albumin as well as across descending tertiles of β_2 -m or albumin-adjusted CC16 in NALF (P -trend = 0.006, 0.02, 0.006 and 0.044, respectively). While the positive associations with urea-adjusted β_2 -m and albumin also emerged with persistent sensitization to HDM or pollen considered separately, the inverse associations with CC16 were largely driven by the persistent sensitization to HDM, which increased in a remarkable dose-dependent manner across descending tertiles of CC16 concentrations, raw or adjusted to urea or β_2 -m (all P -trend = 0.007). It is only after adjustment with β_2 -m that this inverse association with CC16 was also found with persistent sensitization to pollen. By contrast, no significant associations were seen with the raw concentrations of urea, β_2 -m or albumin. The same analyses performed with the odds of remission or new-onset sensitization also did not reveal any significant association with NALF CC16 (See Tables S4 and S5 in Supplementary material). Of note, however, the risks of new-onset sensitization to pollen (but not to HDM) increased with the urea-adjusted levels of albumin (OR highest tertile vs. lowest tertile = 6.64, 95% CI 1.05–130, P -trend = 0.040) while the risk of new-onset sensitization to HDM (but not to pollen) was inversely associated with the unadjusted levels of β_2 -m in NALF (OR lowest vs. highest tertile = 0.17, 95% CI 0.03–0.61, P -trend = 0.02) (Table S4 in Supplementary material). Last, there were no significant correlations between persistent, new-onset or remitted sensitization to pollen, HDM or any aeroallergen and NALF biomarkers at follow-up (results not shown).

To complete our study, we explored by multiple regression analyses the factors that were associated with baseline levels of NALF biomarkers. Table 4 shows the predictors retained in the model at $P < 0.05$ (for all retained predictors, See Table S6 in Supplementary material).

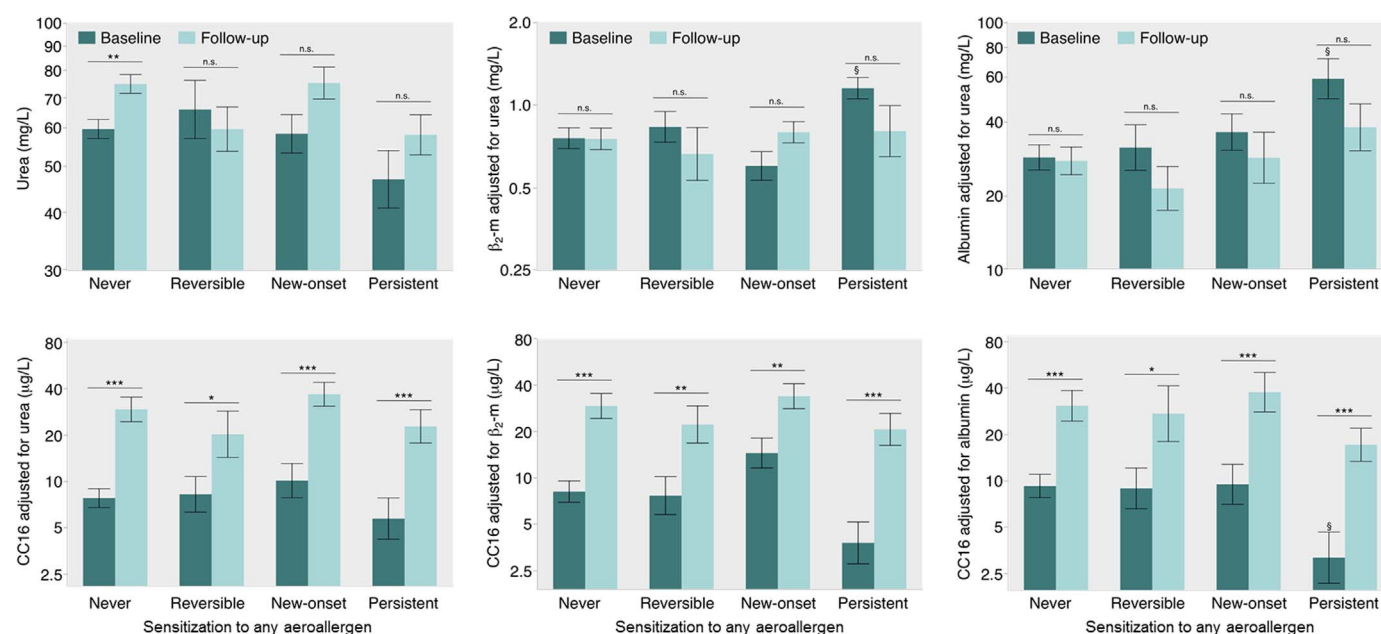


Fig. 1. NALF biomarkers at baseline and follow-up in children with no sensitization, or with reversible, new-onset or persistent sensitization to any aeroallergen. \$significantly different from never-sensitized children.

The strongest and most consistent predictors of NALF biomarkers were house cleaning with bleach and the CPA during the two last years, which both were associated with an increase of urea and a decrease of urea-adjusted CC16. It is worth noting that the negative association with CPA already emerged with the raw concentration of CC16. The CC16 concentration, raw or adjusted, was also inversely associated with asthma, sensitization to HDM, the presence of mould on bedroom wall and the proximity to a busy road. The only statistically significant predictor of increased epithelial permeability was the sensitization to grass pollen, which correlated positively with the unadjusted or adjusted concentrations of albumin and β_2 -m. Excluding children with a diagnosis of respiratory diseases reduced the predictive power of albumin models but increased that of models built with β_2 -m and β_2 -m-adjusted CC16. Fig. 2 illustrates the changes in the covariate-adjusted levels of urea and of urea-adjusted CC16 at baseline according to the CPA and the domestic use of bleach. On average, living in house cleaned with bleach was associated with a 35% increase of urea and a 52% decrease of urea-adjusted CC16 while being in the highest tertile of CPA during the two last years (> 103 h) was associated with a 23% increase of urea and a 45% decrease of urea-adjusted CC16. Excluding children with allergic diseases ($n = 29$) if anything strengthened these associations.

4. Discussion

To the best of our knowledge, we report here the first prospective study exploring the associations between nasal epithelium integrity and the risks of aeroallergen sensitization. Contrary to the intuitive expectation, alterations in epithelial barrier function as assessed by NALF biomarkers were more closely related to the persistence than to the new-onset of allergic sensitization. The risks of persistent sensitization to any aeroallergen, HDM or pollen were dose-dependently associated with increasing urea-adjusted NALF concentrations of β_2 -m or albumin and with decreasing NALF concentrations of CC16, unadjusted or adjusted with β_2 -m or albumin. The risk of new-onset sensitization correlated only with epithelial permeability markers in NALF, positively with the urea-adjusted concentration of albumin in the case of pollen sensitization and negatively with the raw concentration of β_2 -m in the case of HDM sensitization. The strongest and most consistent predictors of NALF biomarkers were the domestic use of bleach and the attendance

of chlorinated pools, which both were associated with higher urea and lower CC16 levels in NALF.

Before interpreting these associations, it is important to consider the determinants of NALF biomarkers that might act as confounders. The biomarker concentration in NALF unavoidably depends on the recovery of ELF, which can be quite variable within and between individuals, especially when examining young children. Classically, the concentration of plasma-derived urea is used as an indicator of the ELF recovery by lavage (Cavaliere et al., 1986). As shown in our study, adjustment with urea indeed improved the accuracy of NALF biomarkers, strengthening for instance the correlations between the baseline and the follow-up levels of albumin or CC16. The problem we unexpectedly encountered is that two of the most consistent predictors of CC16 (CPA and house cleaning with chlorine bleach) were associated with a very significant increase of the urea concentration in NALF. The decrease of urea-adjusted CC16 associated with these two chlorine-related risk factors was, however, about twice larger than this increase of urea. This means that there was really a decrease of CC16 secretion and presumably of CC16-secreting cells in the nasal epithelium of children who had been exposed to CPs. In the case of CPs from swimming pools, this conclusion is supported by the fact that the association with CPA already emerged with the raw concentration of CC16. By contrast, the adjustment with urea could not confound associations of NALF biomarkers with allergic sensitization since the latter showed no relationship at all with the concentration of urea in NALF. Another unexpected finding was that the concentrations of β_2 -m in NALF - like that of urea - was only two to three times lower than that in plasma, a dilution factor, which is certainly one order of magnitude lower than that expected from the instillation of 2.5 ml in each nostril. The only possible explanation for such a small dilution is a building up in the ELF of β_2 -m and urea originating from plasma. Because CC16 has a molecular size close to that of β_2 -m, the leakage of CC16 from plasma into the ELF should entail a NALF concentration about 2–3 fold lower than that in plasma. The mean CC16 concentration in plasma of children being on average 7–10 $\mu\text{g/L}$ (Lagerkvist et al., 2004; Bernard et al., 2003, 2007), the corresponding NALF concentration of plasma-derived CC16 can be estimated at 2–5 $\mu\text{g/L}$, which represents an important part of the NALF concentrations observed at baseline in our study. To avoid confounding by this extra-nasal source of CC16, we adjusted CC16 to β_2 -m as an index correcting NALF CC16 levels for both the ELF recovery

Table 3

Odds ratios (95% CI) of persistent sensitization to any aeroallergen, HDM or pollen in children according to ascending or descending tertiles of NALF biomarkers.

	Any aeroallergen		HDM		Pollen	
	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)
Urea						
Lowest	8 (19.5)	1.00	5 (12.2)	1.00	2 (4.9)	1.00
Middle	4 (10.0)	0.56 (0.14–2.22)	1 (2.5)	0.19 (0.01–1.33)	3 (7.5)	1.92 (0.27–17.4)
Highest	4 (10.0)	0.44 (0.08–1.87)	2 (5.0)	0.46 (0.06–2.50)	2 (5.0)	1.00 (0.10–10.5)
P-trend	0.20		0.19		0.98	
β_2 -m						
Lowest	2 (5.1)	1.00	1 (2.6)	1.00	0 (0)	1.00
Middle	8 (21.0)	5.26 (1.08–40.2)	5 (13.2)	5.60 (0.80–113)	3 (7.9)	3.68 (0.46–77.3)
Highest	5 (13.2)	3.49 (0.62–28.7)	2 (5.3)	2.51 (0.22–58.3)	4 (10.5)	5.38 (0.70–114)
P-trend	0.29		0.63		0.053	
β_2 -m adj for urea						
Lowest	1 (2.6)	1.00	0 (0)	1.00	1 (2.6)	1.00
Middle	5 (13.2)	6.40 (0.89–1.32)	3 (7.9)	4.65 (0.63–94.6)	0 (0)	0.53 (0.02–5.90)
Highest	9 (23.7)	13.9 (2.17–277)	5 (13.2)	7.37 (1.12–146)	6 (15.8)	3.88 (0.82–26.4)
P-trend	0.006		0.02		0.02	
Alb						
Lowest	3 (7.3)	1.00	2 (4.9)	1.00	0 (0)	1.00
Middle	7 (17.5)	4.68 (1.06–26.8)	4 (10.0)	3.37 (0.57–32.4)	3 (7.5)	5.30 (0.69–111)
Highest	6 (15.0)	3.04 (0.67–17.1)	2 (5.0)	1.34 (0.15–12.6)	4 (10)	5.90 (0.83–122)
P-trend	0.30		0.98		0.053	
Alb adjusted for urea						
Lowest	1 (2.4)	1.00	0 (0.0)		1 (2.4)	1.00
Middle	7 (17.5)	10.3 (1.60–205)	4 (10.0)	5.86 (0.87–116)	1 (2.4)	0.97 (0.03–27.0)
Highest	8 (20.0)	12.2 (1.94–241)	4 (10.0)	6.29 (0.92–126)	5 (12.5)	7.26 (0.94–1.58)
P-trend	0.02		0.07		0.053	
CC16						
Highest	3 (7.3)	1.00	0 (0.0)	1.00	2 (4.9)	1.00
Middle	6 (15.0)	2.85 (0.63–16.2)	2 (5.0)	3.96 (0.46–85.7)	2 (5.0)	1.06 (0.11–10.5)
Lowest	7 (17.5)	4.18 (0.94–24.6)	6 (15.0)	13.0 (1.89–273)	3 (7.8)	1.91 (0.25–19.1)
P-trend	0.18		0.007		0.61	
CC16 adjusted for urea						
Highest	3 (7.3)	1.00	0 (0.0)	1.00	2 (5.2)	1.00
Middle	6 (15.0)	3.15 (0.69–18.2)	2 (5.0)	4.05 (0.47–86.6)	2 (5.2)	1.49 (0.15–14.9)
Lowest	7 (17.5)	3.43 (0.79–19.2)	6 (15.0)	9.80 (1.54–195)	3 (7.8)	1.53 (0.20–14.4)
P-trend	0.18		0.007		0.61	
CC16 adjusted for β_2 -m						
Highest	1 (2.6)	1.00	0 (0.0)	1.00	0 (0.0)	1.00
Middle	5 (13.2)	7.99 (1.01–177)	2 (5.3)	3.30 (0.39–69.3)	2 (5.2)	3.27 (0.38–69.4)
Lowest	9 (23.7)	19.6 (2.79–425)	6 (15.4)	9.06 (1.45–177)	5 (13.2)	7.29 (1.09–146)
P-trend	0.006		0.007		0.02	
CC16 adjusted for albumin						
Highest	3 (7.3)	1.00	1 (2.4)	1.00	1 (2.4)	1.00
Middle	4 (10.0)	1.43 (0.27–8.48)	2 (5.0)	2.24 (0.19–51.5)	1 (2.5)	0.96 (0.03–28.4)
Lowest	9 (22.5)	6.00 (1.40–35.0)	5 (12.5)	9.83 (1.27–2.18)	5 (12.5)	16.6 (1.49–656)
P-trend	0.044		0.07		0.053	

Odds ratios were adjusted for gender and parental asthma/allergy. When there was a null value among referents, the odds ratio was calculated by adding 1 to each category.

and the CC16 influx from plasma. This index based on similar small-sized proteins probably allows a more accurate assessment of nasal epithelium integrity, explaining why associations with persistent allergic sensitization are much stronger when CC16 is adjusted to β_2 -m than to urea or albumin.

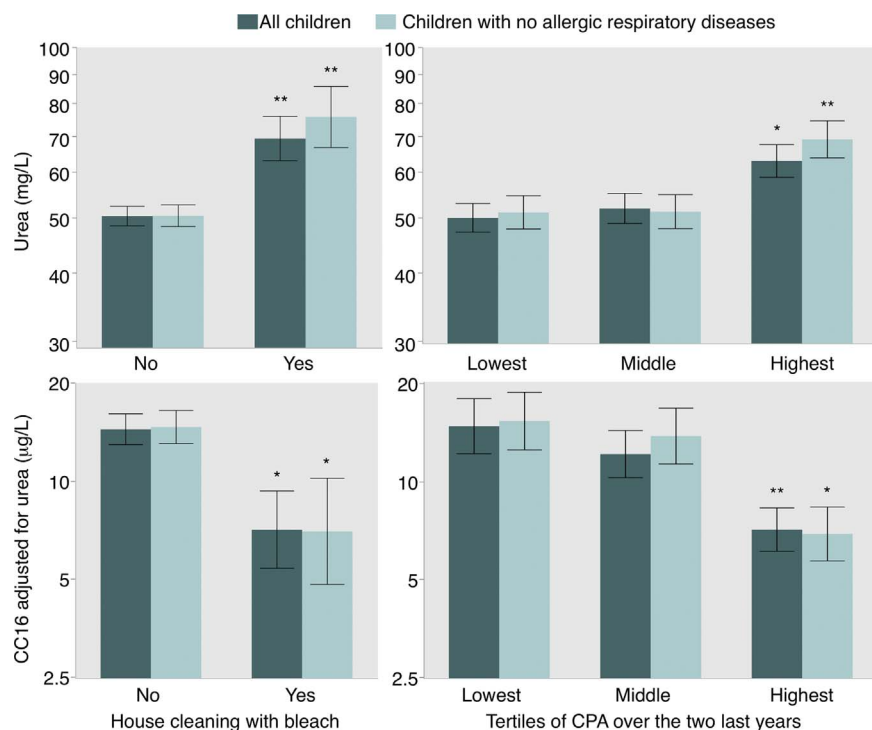
Despite the prospective nature of our study, the interpretation of associations between NALF biomarkers and allergic sensitization is not straightforward. The reason is that NALF biomarkers correlated mostly with persistent sensitization, which leaves open the possibility of reverse causation due to epithelium alterations by aeroallergen exposure or airway inflammation. Five observations, however, argue against this possibility. First, changes of NALF biomarkers during the follow-up (increase of CC16 and decrease of albumin and β_2 -m) were not significantly different between children with persistent sensitization and their peers with new-onset, remitted or no sensitization at all. Second, the correlations of follow-up with baseline levels of albumin and CC16 did not differ meaningfully between children with and those without allergic diseases. Third, in multiple regression analyses a diagnosis of perennial allergic rhinitis or of hay fever, which are diseases frequently associated with upper airway inflammation, was not retained as

determinant of NALF biomarkers or when it was retained (for urea-adjusted CC16) it was in the opposite direction of that observed with persistent sensitization. Fourth, there was no correlation between NALF biomarkers and exhaled NO, a marker of inflammation frequently elevated in patients with allergic rhinitis. Fifth, there were no associations at all between persistent, new-onset or remitted sensitization and NALF biomarkers at follow-up. Considering these elements, the most likely explanation for associations between NALF biomarkers and persistent allergic sensitization is that of a dysfunctional nasal epithelium barrier facilitating the maintenance of allergic sensitization. The strong inverse association between CC16 and persistent aeroallergen sensitization might reflect the promoting effect on sensitization of a decreased secretion of CC16, a multifunctional protein capable of down-regulating the inflammation and the Th2 differentiation both in vitro and in vivo (Hung et al., 2004; Johansson et al., 2007; Tokita et al., 2014). As to the positive associations between persistent sensitization and β_2 -m or albumin in NALF, they seem to have different meanings according to the size of protein and the nature of the allergen. In the case of pollen, the positive association with β_2 -m or albumin in NALF can be interpreted as the evidence that maintenance of sensitization is facilitated by an

Table 4Determinants of NALF biomarker at baseline in all children or in children with no diagnosis of respiratory allergic diseases ($P < 0.05$).

Dependent variable	All children (n = 121)			Children with no respiratory allergic diseases (92)		
	Independent variable	Coefficient (95% CI)	P	Independent variable	Coefficient (95% CI)	P
Log urea	House cleaning with bleach	0.13 (0.05–0.22)	0.002	Highest tertile of recent CPA	0.13 (0.05–0.20)	0.001
	Highest tertile of recent CPA	0.09 (0.03–0.16)	0.007	House cleaning with bleach	0.17 (0.06–0.27)	0.002
Log β_2 -m	Grass pollen sensitization	0.20 (0.00005–0.39)	0.049	House cleaning with bleach	0.22 (0.04–0.40)	0.02
				Grass pollen sensitization	0.29 (0.02–0.57)	0.03
Log β_2 -m, urea-adjusted	Grass pollen sensitization	0.24 (0.08–0.41)	0.005	Tree pollen sensitization	0.27 (0.02–0.52)	0.04
				Grass pollen sensitization	0.27 (0.03–0.51)	0.03
Log Alb	Mother with asthma or allergy	−0.20 (−0.37 to −0.03)	0.02	–	–	–
Log Alb, urea-adjusted	House cleaning with bleach	−0.26 (−0.44 to −0.08)	0.004	House cleaning with bleach	−0.25 (−0.49 to −0.003)	0.048
	Grass pollen sensitization	0.28 (0.03–0.53)	0.03			
Log CC16	Mother with asthma or allergy	−0.16 (−0.31 to −0.006)	0.041			
	Mould on bedroom wall	−0.47 (−0.89 to −0.06)	0.03	Living < 100 from a busy road	−0.23 (−0.42 to −0.04)	0.02
	Living < 100 from a busy road	−0.18 (−0.35 to −0.01)	0.03	Mould on bedroom wall	−0.41 (−0.87 to 0.05)	0.03
	Highest tertile of recent CPA	−0.18 (−0.36 to −0.004)	0.01			
	Perennial allergic rhinitis	0.28 (0.002–0.56)	0.049			
Log CC16, urea-adjusted	House cleaning with bleach	−0.32 (−0.57 to −0.09)	0.007	Highest tertile of recent CPA	−0.32 (−0.54 to −0.10)	0.004
	Highest tertile of recent CPA	−0.26 (−0.45 to −0.07)	0.008	Living < 100 from a busy road	−0.25 (−0.45 to −0.05)	0.01
	Asthma	−0.36 (−0.69 to 0.04)	0.03	House cleaning with bleach	−0.33 (−0.63 to −0.03)	0.03
	Perennial allergic rhinitis	0.33 (0.03–0.62)	0.03	Mould on bedroom wall	−0.54 (−1.03 to −0.05)	0.03
Log CC16, β_2 -m-adjusted	HDM sensitization	−0.36 (−0.61 to −0.10)	0.007	House cleaning with bleach	−0.41 (−0.71 to −0.12)	0.007
				Mould on bedroom wall	−0.55 (−1.03 to −0.07)	0.03
				Tree pollen sensitization	−0.45 (−0.85 to −0.05)	0.03
				Living < 100 from a busy road	−0.22 (−0.42 to −0.02)	0.040
Log CC16, Alb-adjusted				Grass pollen sensitization	−0.46 (−0.89 to −0.03)	0.040
				Highest tertile of recent CPA	−0.22 (−0.44 to −0.008)	0.042
	Mother with asthma or allergy	0.29 (0.06–0.52)	0.02	Grass pollen sensitization	−0.57 (−1.1 to −0.04)	0.03
	Age (years)	0.41 (0.07–0.75)	0.02			
	HDM sensitization	−0.32 (−0.61 to −0.02)	0.03			

CPA tertiles, hours: All children (children with no allergic diseases) lowest, 0–29 (0–28); middle, 30–100 (29–100); highest, 103–588 (103–588).

**Fig. 2.** NALF concentrations of urea and of urea-adjusted CC16 in children stratified according to house cleaning with bleach or according to tertiles of cumulated chlorinated pool attendance (cumulated hours) during the two last years. *denotes statistical difference by comparison with children not living in a house cleaned with bleach or children in the lowest tertile of chlorinated pool attendance.

increased epithelial permeability, which quite conceivably would enhance the delivery of allergens to dendritic cells. Because most allergens have a molecular weight between 5 and 150 kDa (Alessandri et al., 2009), a range largely covered by the molecular weights of β_2 -m and albumin, they probably follow - but in the opposite direction - the same paracellular routes as these two plasma proteins. The alternative explanation of a disruption of epithelial barrier caused by the pollen proteolytic activity appears less likely if one considers the transient

nature of epithelial barrier disruption after pollen challenge (Steelant et al., 2016) and the fact that our children were examined outside the pollination periods. By contrast, the positive association between urea-adjusted β_2 -m and persistent HDM sensitization most probably reflect a slight epithelial barrier disruption by the proteolytic activity of this perennial allergen, an interpretation that can explain the opposite association between urea-adjusted β_2 -m and new-onset HDM sensitization.

Our study provides insight into the factors influencing nasal epithelium integrity. By far the most influential factor is the age since within a period of only two years the NALF concentration of CC16 of children was multiplied by a factor of four. If one takes into account that at this young age more than 50% of NALF CC16 probably originates from plasma, it can be estimated that the amount of CC16 secreted by the nasal epithelium has increased by one order of magnitude during the follow-up. This testifies to the great immaturity of the nasal epithelium of young children, which therefore might be particularly sensitive to stressors. Of the stressors identified in our study, one of the strongest and most consistent is the child inhalation exposure to CPs in the form of chlorine gas released by bleach or of hypochlorite/hypochlorous acid, chloramines and other irritant CPs in swimming pool environment (Bernard, 2007). The decrease of CC16 in NALF associated with the exposure to these CPs might be the reflection of damaged CC16-secreting cells in nasal epithelium. Another possibility is a transdifferentiation of these CC16-secreting cells into ciliated cells or mucus-secreting goblet cells (Cui et al., 2011), which would be triggered as a defence mechanism against the irritating effects of CPs. This defence mechanism might also explain the increased levels of NALF urea associated with CPs exposure. These observations confirm our previous cross-sectional studies among children or adolescents showing that chlorinated attendance is associated with decreased levels of CC16 in serum or NALF (Lagerkvist et al., 2004; Cui et al., 2011). Our findings also concord with recent studies showing that the frequent use of bleach and other products for home cleaning is associated with an increased risk of rhinitis and in general of respiratory problems in children (Bernard et al., 2007; Liu et al., 2016; Casas et al., 2013, 2015). By contrast, we found very few predictors of albumin and β_2 -m in NALF which are biomarkers of epithelial permeability. The only consistent one was the association of raw or urea-adjusted β_2 -m with sensitization to pollen, in particular with grass pollen, but as discussed above the sensitization is most likely the consequence than the cause of increased epithelial permeability.

Our study presents two major limitations. The first is the relatively small size of the cohort due to the difficulties to perform longitudinal studies in young schoolchildren. Apart from reducing the statistical power of our study, we do not think that loss during follow-up has biased our observations as there were no meaningful differences between participants and non-participants regarding most outcomes and risk factors. The second limitation is the lack of exposure data for outdoor or indoor pollutants whose effects have certainly been underestimated. This is probably the case of traffic-related air pollutants, which were significantly associated with lower NALF CC16 despite of use a proxy measure such as the distance to a busy road.

In conclusion, a defective nasal epithelium in children as reflected by low CC16 and high albumin levels in NALF is predictive of more persistent sensitization to aeroallergen and especially to HDM. The immaturity of the nasal epithelium during early childhood along with the exposure to environmental stressors such as CPs appear to be largely responsible for these functional deficits in the nasal epithelial barrier function.

Author contributions

A.B. wrote the manuscript and provided the scientific guidance of the project, participating to the study design, analysis of data and interpretation of the results. C.V., A.S. and X.D. recruited the children, organized their examination in schools, collected the information from the questionnaires and interviews and performed the analyses of biomarkers.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.envres.2017.06.009>.

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Supplementary material

Table S1. Independent variables tested in multiple regression analyses

1. Age (years)
2. Gender (boy, 1)
3. Log BMI (kg/m²)
4. Mother with asthma and/or allergies, yes/no
5. Father with asthma and/or allergies, yes/no
6. Log birth weight (g)
7. Season of birth (summer, winter)
8. Parental smoking (parents or relatives smoking at home) (yes/no)
9. Maternal smoking during pregnancy (yes/no)
10. Living at <100 m from a busy road (yes/no)
11. Day care attendance (yes/no)
12. Breastfeeding (yes/no)
13. Ever asthma (diagnosed by a physician), yes/no
14. Ever perennial allergic rhinitis (diagnosed by a physician), yes/no
15. Ever hay fever (diagnosed by a physician), yes/no
16. Frequent cold (yes/no)
17. Medication for asthma or allergic diseases (yes/no)
18. Furry pets at home (yes/no)
19. Mould on bedroom wall (yes/no)
21. Older siblings (yes/no)
22. House cleaning with bleach (yes/no)

23. Cumulative pool attendance (CPA) over the two last years (tertiles of the total number of hours tested as dummy variables)

24. Cumulative pool attendance (CPA) over lifetime (tertiles of the total number of hours tested as dummy variables)

Table S2. Correlations between NALF biomarkers at baseline and follow-up

		Urea	β_2 -m	β_2 -m adj. for urea	Alb	Alb adj. for urea	CC16	CC16 adj. for urea	CC16 adj. for β_2 -m
β_2 -m	Baseline	0.51***							
	Follow-up	0.33***							
β_2 -m adj. for urea	Baseline	-0.09	0.81***						
	Follow-up	-0.18*	0.87***						
Alb	Baseline	0.36***	0.36***	0.18					
	Follow-up	0.28**	0.35***	0.22*					
Alb adj. for urea	Baseline	-0.08	0.15	0.23*	0.90***				
	Follow-up	-0.09	0.23*	0.30**	0.93***				
CC16	Baseline	-0.02	0.12	0.15	0.08	0.09			
	Follow-up	0.18*	0.42***	0.34***	0.10	0.04			
CC16 adj. for urea	Baseline	-0.38***	-0.06	0.17	-0.06	0.11	0.93***		
	Follow-up	-0.11	0.32***	0.39***	0.02	0.07	0.96***		
CC16 adj. for β_2 -m	Baseline	-0.31***	-0.47***	-0.33***	-0.14	-0.01	0.87***		
	Follow-up	-0.02	-0.19*	-0.19*	-0.12	-0.11	0.81***		
CC16 adj. for Alb	Baseline	-0.27***	-0.15	-0.005	-0.63***	-0.55***	0.72***	0.76***	0.73***
	Follow-up	-0.03	0.11	0.13	-0.57***	-0.58***	0.76***	0.77***	0.75***

Table S3. Correlation between NALF biomarkers at follow-up and at baseline in all children (n=121) in children without or with a diagnosis of allergic diseases at baseline

Biomarker	All children				Children with no allergic diseases				Children with allergic diseases			
	n	r	Equation	P	n	r	Equation	P	n	r	Equation	P
Log urea, mg/l	121	0.05	$y = 1.77 + 0.05 x$	0.55	92	0.08	$y = 1.71 + 0.75 x$	0.43	29	-0.02	$y = 1.91 + 0.02 x$	0.92
Log β_2 -m, μ g/l	114	0.05	$y = 2.72 + 0.06 x$	0.60	86	0.04	$y = 1.24 + 0.18x$	0.73	28	0.10	$y = 1.25 + 0.26 x$	0.62
Log β_2 -m adj. for urea (μ g/l)	114	0.14	$y = 2.37 + 0.18 x$	0.13	86	0.15	$y = 2.34 + 0.19 x$	0.18	28	0.12	$y = 2.47 + 0.14 x$	0.54
Log Alb (mg/l)	121	0.31	$y = 0.93 + 0.34 x$	<0.001	92	0.25	$y = 1.02 + 0.26 x$	0.01	29	0.48	$y = 0.58 + 0.59 x$	0.009
Log Alb adj. for urea	121	0.39	$y = 0.78 + 0.43 x$	<0.001	92	0.34	$y = 0.89 + 0.35 x$	<0.001	29	0.51	$y = 0.36 + 0.72 x$	0.005
Log CC16 (μ g/l)	121	0.16	$y = 1.25 + 0.20 x$	0.07	92	0.14	$y = 1.24 + 0.18 x$	0.17	29	0.20	$y = 1.25 + 0.26 x$	0.20
Log CC16 adj. for urea	121	0.22	$y = 1.20 + 0.25 x$	0.01	92	0.22	$y = 1.18 + 0.25 x$	0.040	29	0.25	$y = 1.26 + 0.22 x$	0.18
Log CC16 adj. for β_2 -m	114	0.32	$y = 1.14 + 0.32 x$	<0.001	86	0.33	$y = 1.08 + 0.35 x$	0.002	28	0.34	$y = 1.28 + 0.23 x$	0.08
Log CC16 adj. for Alb	121	0.35	$y = -1.08 + 0.40 x$	<0.001	92	0.31	$y = 1.10 + 0.37 x$	0.003	29	0.49	$y = 1.00 + 0.48 x$	0.008

Table S4. Odds Ratios (95% CI) of remitted sensitization to any aeroallergen, HDM or pollen in children according to ascending or descending tertiles of NALF biomarkers

	Any aeroallergen		HDM		Pollen	
	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)
Urea						
Lowest	11 (26.8)	1.00	2 (4.9)	1.00	6 (14.6)	1.00
Middle	11 (27.5)	1.05 (0.38-2.87)	5 (12.5)	2.92 (0.58-21.5)	9 (22.5)	1.73 (0.56-5.75)
Highest	9 (22.5)	0.89 (0.31-2.53)	5 (12.5)	3.04 (0.59-22.8)	5 (12.5)	0.88 (0.23-3.26)
<i>P</i> -trend		0.66		0.25		0.80
β_2 -m						
Lowest	6 (15.4)	1.00	3 (7.7)	1.00	2 (5.1)	1.00
Middle	13 (34.2)	3.21 (1.06-10.7)	3 (7.9)	0.96 (0.16-5.63)	11 (29.0)	7.81 (1.86-53.9)
Highest	11 (29.0)	3.05 (0.96-10.7)	5 (13.2)	1.93 (0.41-10.6)	6 (15.8)	3.87 (0.80-28.5)
<i>P</i> -trend		0.17		0.42		0.20
β_2 -m adj for urea						
Lowest	5 (12.8)	1.00	3 (7.7)	1.00	3 (7.7)	1.00
Middle	14 (36.8)	4.07 (1.33-14.3)	5 (13.2)	1.85 (0.42-9.73)	7 (18.4)	2.72 (0.68-13.5)
Highest	11 (29.0)	3.18 (0.99-11.6)	3 (7.9)	0.97 (0.16-5.69)	9 (23.7)	3.86 (1.02-18.9)
<i>P</i> -trend		0.10		0.97		0.06
Alb tertiles						
Lowest	11 (26.8)	1.00	4 (9.8)	1.00	4 (9.8)	1.00
Middle	9 (22.5)	0.96 (0.33-2.79)	3 (7.5)	0.83 (0.15-4.20)	9 (22.5)	3.27 (0.92-13.6)
Highest	11 (27.5)	1.24 (0.44-3.55)	5 (12.5)	1.40 (0.33-6.35)	7 (17.5)	2.19 (0.58-9.38)
<i>P</i> -trend		0.41		0.68		0.34
Alb adjusted for urea						
Lowest	8 (19.5)	1.00	3 (7.3)	1.00	4 (9.8)	1.00
Middle	12 (30.0)	2.09 (0.73-6.29)	5 (12.5)	1.90 (0.42-10.0)	8 (20.0)	1.66 (0.47-6.21)
Highest	11 (27.5)	1.79 (0.61-5.49)	4 (10.0)	1.42 (0.29-7.79)	8 (20.0)	1.87 (0.55-6.90)
<i>P</i> -trend		0.41		0.68		0.21
CC16						
Highest	8 (19.5)	1.00	2 (4.9)	1.00	5 (12.2)	1.00
Middle	15 (37.5)	2.69 (0.98-7.90)	9 (22.5)	1.86 (1.37-40.8)	9 (22.5)	2.14 (0.66-7.68)
Lowest	8 (20.0)	1.13 (0.36-3.53)	1 (2.5)	0.52 (0.02-5.78)	6 (15.0)	1.35 (0.37-5.16)
<i>P</i> -trend		0.95		0.74		0.73
CC16 adjusted for urea						
Highest	10 (24.4)	1.00	3 (7.3)	1.00	6 (14.6)	1.00
Middle	11 (27.5)	1.16 (0.42-3.26)	7 (17.5)	3.02 (0.75-15.5)	7 (17.5)	1.26 (0.38-4.37)
Lowest	10 (25.0)	1.07 (0.38-3.05)	2 (5.0)	0.66 (0.08-4.25)	7 (17.5)	1.25 (0.37-4.29)
<i>P</i> -trend		0.95		0.74		0.73
CC16 adjusted for β_2 -m						
Highest	8 (20.5)	1.00	3 (7.7)	1.00	3 (7.7)	1.00
Middle	13 (31.6))	1.91 (0.66-5.77)	5 (13.2)	1.79 (0.40-9.51)	10 (26.3)	4.39 (1.18-21.4)
Lowest	10 (26.3)	1.48 (0.50-5.55)	3 (7.9)	1.02 (0.17-5.97)	6 (15.8)	2.31 (0.55-11.9)
<i>P</i> -trend		0.56		0.97		0.33
CC16 adjusted for Alb						
Highest	9 (23.1)	1.00	4 (9.8)	1.00	6 (14.6)	1.00
Middle	10 (26.3)	1.06 (0.38-2.93)	3 (7.5)	0.73 (0.13-3.57)	7 (17.5)	1.21 (0.36-4.18)
Lowest	11 (29.0)	0.87 (0.30-2.50)	5 (12.5)	1.39 (0.33-6.24)	7 (17.5)	1.29 (0.38-4.52)
<i>P</i> -trend		0.56		0.68		0.73

Odds ratios were adjusted for gender and parental asthma/allergy

Table S5. Odds Ratios (95% CI) of new-onset sensitization to any aeroallergen, HDM or pollen in children according to ascending or descending tertiles of NALF biomarkers

	Any aeroallergen		HDM		Pollen	
	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)	N (%)	Adjusted OR (95% CI)
Urea						
Lowest	14 (34.2)	1.00	9 (22.2)	1.00	5 (12.2)	1.00
Middle	8 (20.0)	0.48 (0.17-1.30)	8 (20.0)	0.89 (0.30-2.61)	0 (0)	0.00
Highest	13 (32.5)	0.90 (0.35-2.13)	7 (17.5)	0.72 (0.23-2.19)	5 (12.5)	0.96 (0.24-3.82)
P-trend		0.86		0.62		0.97
β_2 -m						
Lowest	13 (33.3)	1.00	12 (31.0)	1.00	3 (7.7)	1.00
Middle	14 (36.8)	1.09 (0.42-2.84)	8 (21.1)	0.57 (0.19-1.61)	4 (10.5)	1.35 (0.27-7.38)
Highest	6 (15.8)	0.32 (0.10-0.97)	3 (7.9)	0.17 (0.03-0.61)	3 (7.9)	0.89 (0.15-5.27)
P-trend		0.09		0.01		0.97
β_2 -m adj for urea						
Lowest	16 (41.0)	1.00	13 (33.3)	1.00	5 (12.8)	1.00
Middle	8 (21.1)	0.39 (0.14-1.05)	4 (10.5)	0.24 (0.06-0.76)	3 (7.9)	0.60 (0.12-2.69)
Highest	9 (23.7)	0.42 (0.15-1.12)	6 (15.8)	0.36 (0.07-1.07)	2 (5.3)	0.34 (0.05-1.74)
P-trend		0.09		0.053		0.24
Alb tertiles						
Lowest	10 (24.4)	1.00	6 (14.6)	1.00	2 (4.9)	1.00
Middle	11 (27.5)	1.18 (0.42-3.34)	9 (22.5)	1.65 (0.52-5.51)	2 (5.0)	0.99 (0.11-8.84)
Highest	14 (35.0)	1.63 (0.61-4.49)	9 (22.5)	1.69 (0.52-5.66)	6 (15.0)	3.16 (0.65-23.2)
P-trend		0.29		0.37		0.10
Alb adjusted for urea						
Lowest	8 (19.5)	1.00	7 (17.1)	1.00	1 (2.4)	1.00
Middle	13 (32.5)	1.97 (0.71-5.75)	8 (20.0)	1.15 (0.36-3.69)	3 (7.5)	3.32 (0.40-69.5)
Highest	14 (35.0)	2.16 (0.79-6.25)	9 (22.5)	1.38 (0.45-4.37)	6 (15.0)	6.64 (1.05-130)
P-trend		0.12		0.54		0.040
CC16						
Highest	14 (34.2)	1.00	11 (26.8)	1.00	4 (9.8)	1.00
Middle	11 (27.5)	0.71 (0.27-1.85)	6 (15.0)	0.47 (0.15-1.41)	4 (10.0)	0.97 (0.21-4.42)
Lowest	10 (25.0)	0.63 (0.23-1.65)	7 (17.5)	0.56 (0.18-1.62)	2 (5.0)	0.46 (0.06-2.51)
P-trend		0.36		0.29		0.44
CC16 adjusted for urea						
Highest	13 (31.7)	1.00	10 (24.4)	1.00	4 (9.8)	1.00
Middle	8 (20.0)	0.56 (0.19-1.53)	5 (12.5)	0.45 (0.13-1.42)	2 (5.0)	0.48 (0.06-2.66)
Lowest	14 (35.0)	1.13 (0.44-2.89)	9 (22.5)	0.89 (0.31-2.54)	4 (10)	0.95 (0.21-4.38)
P-trend		0.75		0.82		0.97
CC16 adjusted for β_2 -m						
Highest	14 (35.9)	1.00	11 (28.2)	1.00	6 (15.4)	1.00
Middle	11 (29.0)	0.68 (0.25-1.81)	7 (18.4)	0.56 (0.18-1.66)	2 (5.3)	0.27 (0.04-1.29)
Lowest	8 (21.1)	0.44 (0.15-1.22)	5 (13.2)	0.38 (0.11-1.18)	2 (5.3)	0.26 (0.04-1.28)
P-trend		0.15		0.10		0.12
CC16 adjusted for Alb						
Highest	12 (29.3)	1.00	9 (22.0)	1.00	2 (4.9)	1.00
Middle	10 (25.0)	0.77 (0.28-2.09)	6 (15.0)	0.62 (0.19-1.95)	5 (12.5)	2.61 (0.51-19.3)
Lowest	13 (32.5)	1.13 (0.43-2.98)	9 (22.5)	1.01 (0.34-2.97)	3 (7.5)	1.44 (0.22-11.6)
P-trend		0.24		0.96		0.66

Odds ratios were adjusted for gender and parental asthma/allergy.

Table S6 Determinants of NALF CC16 levels at baseline in all children

Dependent variable	Independent variable	Coefficient (95% CI)	P	R ²
Log urea, mg/l	House cleaning with bleach	0.13 (0.05 to 0.22)	0.002	0.159
	Highest tertile of recent CPA	0.09 (0.03 to 0.16)	0.007	
	Log BMI	-0.43 (-0.91 to 0.05)	0.08	
	Smoking during pregnancy	0.08 (-0.02 to 0.17)	0.10	
Log β_2 -m, mg/l,	Grass pollen sensitization	0.20 (0.00005 to 0.39)	0.049	0.127
	Tree pollen sensitization	0.17 (-0.02 to 0.35)	0.08	
	House cleaning with bleach	0.13 (-0.01 to 0.27)	0.08	
	Smoking during pregnancy	0.14 (-0.03 to 0.30)	0.11	
	Mother with asthma or allergy	-0.10 (-0.22 to 0.03)	0.12	
Log β_2 -m, mg/l, adjusted for urea	Grass pollen sensitization	0.24 (0.08 to 0.41)	0.005	0.11
	Tree pollen sensitization	0.17 (-0.02 to 0.35)	0.08	
	Age (years)	0.12 (-0.04 to 0.27)	0.13	
Log Alb, mg/l	Mother with asthma or allergy	-0.20 (-0.37 to -0.03)	0.02	0.124
	Breastfeeding	0.20 (-0.006 to 0.40)	0.057	
	Tree pollen sensitization	0.23 (-0.03 to 0.48)	0.08	
	House cleaning with bleach	-0.16 (-0.35 to 0.04)	0.11	
	Grass pollen sensitization	0.21 (-0.07 to 0.48)	0.14	
Log Alb, mg/l, adjusted for urea	House cleaning with bleach	-0.26 (-0.44 to -0.08)	0.004	0.205
	Grass pollen sensitization	0.28 (0.03 to 0.53)	0.03	
	Mother with asthma or allergy	-0.16 (-0.31 to -0.006)	0.041	
	Tree pollen sensitization	0.23 (-0.01 to 0.46)	0.06	
	Highest tertile of recent CPA	-0.12 (-0.26 to 0.03)	0.11	
	Frequent cold	0.11 (-0.03 to 0.25)	0.12	
	Living<100 from a busy road	-0.11 (-0.24 to 0.03)	0.12	
	Breastfeeding	0.14 (-0.04 to 0.33)	0.12	
Log CC16, μ g/l	Mould on bedroom wall	-0.47 (-0.89 to -0.06)	0.03	0.165
	Living<100 from a busy road	-0.18 (-0.35 to -0.01)	0.03	
	Highest tertile of recent CPA	-0.18 (-0.36 to -0.004)	0.01	
	Allergic rhinitis	0.28 (0.002 to 0.56)	0.049	
	HDM sensitization	-0.22 (-0.45 to 0.004)	0.053	
	Smoking during pregnancy	0.24 (-0.01 to 0.49)	0.06	
	Asthma	-0.26 (-0.57 to 0.04)	0.09	
	House cleaning with bleach	-0.18 (-0.40 to 0.04)	0.11	
Log CC16, μ g/l, adjusted for urea	House cleaning with bleach	-0.32 (-0.57 to -0.09)	0.007	0.181
	Highest tertile of recent CPA	-0.26 (-0.45 to -0.07)	0.008	
	Asthma	-0.36 (-0.69 to 0.04)	0.03	
	Allergic rhinitis	0.33 (0.03 to 0.62)	0.03	
	Living<100 from a busy road	-0.18 (-0.35 to 0.0005)	0.051	
	Mould on bedroom wall	-0.42 (-0.86 to 0.03)	0.07	
	HDM sensitization	-0.20 (-0.45 to 0.04)	0.10	
	Age (years)	0.22 (-0.06 to 0.49)	0.13	
Log CC16, μ g/l, adjusted for β_2 -m	HDM sensitization	-0.36 (-0.61 to -0.10)	0.007	0.118
	Living<100 from a busy road	-0.15 (-0.34 to 0.04)	0.11	
	Mother with asthma or allergy	0.17 (-0.04 to 0.38)	0.11	
	House cleaning with bleach	-0.19 (-0.43 to 0.06)	0.14	
Log CC16, μ g/l, adjusted for Alb	Mother with asthma or allergy	0.29 (0.06 to 0.52)	0.02	0.186
	Age (years)	0.41 (0.07 to 0.75)	0.02	
	HDM sensitization	-0.32 (-0.61 to -0.02)	0.03	
	Asthma	-0.33 (-0.71 to 0.05)	0.09	
	Father with asthma or allergy	0.24 (-0.04 to 0.52)	0.09	
	Tree pollen sensitization	-0.32 (-0.70 to 0.06)	0.10	
	Parental smoking	0.21 (-0.04 to 0.46)	0.10	
	Frequent cold	-0.17 (-0.39 to 0.04)	0.12	

Table S7 Determinants of baseline levels NALF biomarkers in children with no diagnosis of asthma, allergic rhinitis or hay fever

Dependent variable	Independent variable	Coefficient (95% CI)	P	R ²
Log urea, mg/l	Highest tertile of recent CPA	0.13 (0.05 to 0.20)	0.001	0.176
	House cleaning with bleach	0.17 (0.06 to 0.27)	0.002	
Log β_2 -m, μ g/l,	House cleaning with bleach	0.22 (0.04 to 0.40)	0.02	0.198
	Grass pollen sensitization	0.29 (0.02 to 0.57)	0.03	
	Tree pollen sensitization	0.27 (0.02 to 0.52)	0.04	
	Mother with asthma or allergy	-0.14 (-0.29 to 0.004)	0.057	
	Middle tertile of recent CPA	-0.12 (-0.25 to 0.02)	0.09	
	Older siblings	0.10 (-0.03 to 0.23)	0.12	
Log β_2 -m, μ g/l, adjusted for urea	Grass pollen sensitization	0.27 (0.03 to 0.51)	0.03	0.078
	Older siblings	0.09 (-0.03 to 0.20)	0.14	
Log Alb, mg/l	Boy	0.15 (-0.02 to 0.33)	0.09	0.064
	Breastfeeding	0.18 (-0.05 to 0.41)	0.13	
Log Alb, mg/l, adjusted for urea	House cleaning with bleach	-0.25 (-0.49 to -0.003)	0.048	0.080
	Grass pollen sensitization	0.28 (-0.08 to 0.64)	0.12	
	Highest tertile of recent CPA	-0.14 (-0.31 to 0.04)	0.13	
Log CC16, μ g/l	Living<100 from a busy road	-0.23 (-0.42 to -0.04)	0.02	0.148
	Mould on bedroom wall	-0.41 (-0.87 to 0.05)	0.03	
	Age (years)	0.24 (-0.07 to 0.55)	0.13	
	Highest tertile of recent CPA	-0.15 (-0.36 to 0.05)	0.14	
Log CC16, μ g/l, adjusted for urea	Highest tertile of recent CPA	-0.32 (-0.54 to -0.10)	0.004	0.143
	Living<100 from a busy road	-0.25 (-0.45 to -0.05)	0.01	
	House cleaning with bleach	-0.33 (-0.63 to -0.03)	0.03	
	Mould on bedroom wall	-0.54 (-1.03 to -0.05)	0.03	
Log CC16, μ g/l, adjusted for β_2 -m	House cleaning with bleach	-0.412 (-0.71 to -0.12)	0.007	0.250
	Mould on bedroom wall	-0.550 (-1.03 to -0.07)	0.03	
	Tree pollen sensitization	-0.449 (-0.85 to -0.05)	0.03	
	Living<100 from a busy road	-0.217 (-0.42 to -0.02)	0.040	
	Grass pollen sensitization	-0.462 (-0.89 to -0.03)	0.040	
	Highest tertile of recent CPA	-0.224 (-0.44 to -0.008)	0.042	
Log CC16, μ g/l, adjusted for Alb	Grass pollen sensitization	-0.57 (-1.1 to -0.04)	0.03	0.153
	Tree pollen sensitization	-0.47 (-0.94 to 0.01)	0.055	
	Mother with asthma or allergy	0.27 (-0.09 to 0.55)	0.058	
	Age (years)	0.36 (-0.02 to 0.74)	0.06	

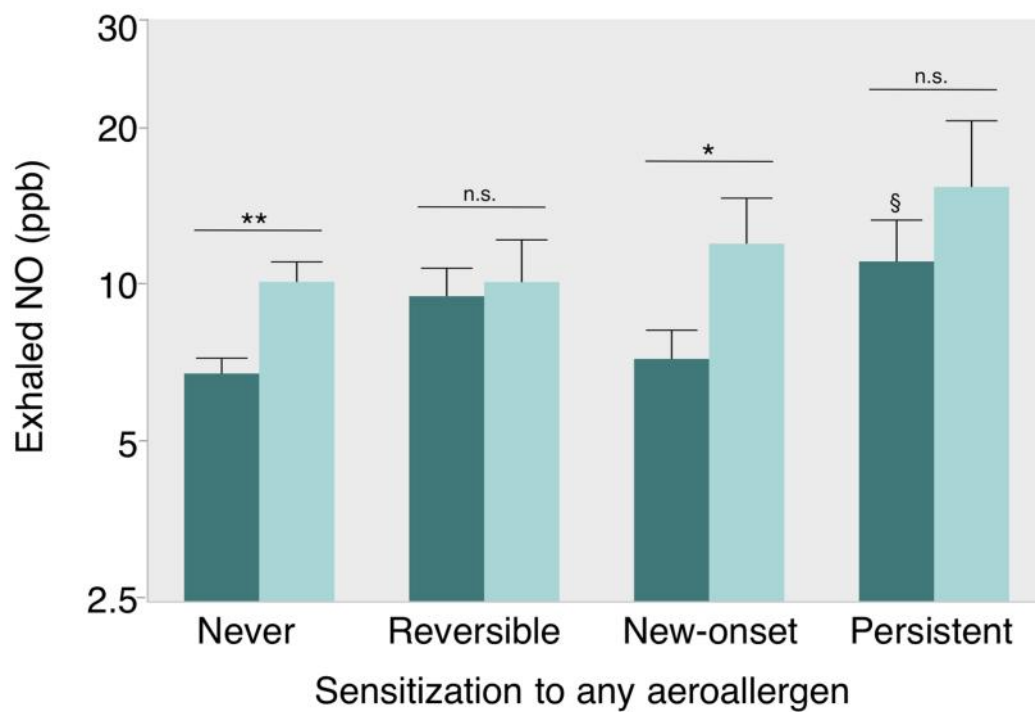


Figure S1. Exhaled NO at baseline and follow-up in children with no sensitization, or with reversible, new-onset or persistent sensitization to any aeroallergen. * $P < 0.05$ and ** $P < 0.01$ denote values significantly different from baseline values. §denotes values significantly different from that of never-sensitized children ($P < 0.05$).