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NASAL EPITHELIUM BIOMARKERS IN CHILDREN AND ADOLESCENTS: ASSOCIATIONS WITH ALLERGIC SENSITIZATION AND ENVIRONMENTAL STRESSORS

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Thesis presented with a view to obtaining the degree of Doctor in Biomedical Sciences

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Summary

In this project we have used biomarkers to investigate the effects of environmental stressors, to which we are daily exposed, on the nasal epithelium integrity and explored the possible associations between nasal epithelium changes and the development of allergic sensitization. The integrity of the nasal epithelium of children was assessed non-invasively by measuring two proteins in nasal lavage fluid (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. The second protein is the 16 kDa Clara cell protein, an anti-inflammatory protein secreted in large amounts by the nasal epithelium, which increasingly appears to play a central role in the development of asthma and allergic diseases. We conducted two cross-sectional studies on pediatric populations: the first among 474 secondary-school adolescents (mean age, 15.5 yrs; girls N=263, boys N=211) and the second among 288 kindergarten children (mean age, 5.7; girls N=136; boys N=152).

In the study on adolescents, we measured albumin (Alb) and Clara cell protein (CC16) in NALF from each nostril of subjects who had no signs of rhinitis and were not under medication for asthma or allergies. We adjusted the concentrations of Alb and CC16 in NALF for the serum/NALF urea ratio and calculated the means and ratio of CC16 and Alb. We also measured total and aeroallergen-specific IgE in serum. Stepwise and multiple regression analyses were used. Attendance at indoor chlorinated pools was the only factor influencing, both negatively, CC16 levels and the CC16/Alb ratio in NALF (both P=0.01). Active smoking decreased CC16 in NALF (P=0.005) but had no influence on the CC16/Alb ratio. These factors did not affect Alb in NALF which varied only with age and sex. Among adolescents with serum IgE>30 kUI/l, those in the lowest quartile of CC16 or of the

CC16/Alb ratio in NALF had greater risk of sensitization to aeroallergens as compared to subjects in the highest quartiles (adjusted ORs 2.16, 95th CI 1.05-4.41 and 2.52, 95th CI 1.09-5.83, respectively). These associations persisted when excluding adolescents with doctor-diagnosed hay fever or allergic rhinitis.

In the study among kindergarten children, we measured aeroallergen-specific IgE in nasal mucosa and the concentrations of urea, albumin and Clara cell protein (CC16) in NALF. Albumin and CC16 were expressed per liter or as a ratio to urea. We also calculated the CC16/albumin ratio as an index integrating the permeability and the secretory function of the nasal epithelium. The median NALF concentration of albumin (34.5 mg/l) in children was three times higher than in adolescents while that of CC16 (8.2 μg/l) and the CC16/albumin ratio were respectively three and 10 times lower. Median NALF concentrations of CC16 and albumin were 8.2 μg/l and 34.5 mg/l, respectively. While there were no significant gender differences when proteins were expressed per liter, the NALF CC16/albumin ratio was significantly higher in girls than in boys and a trend for higher values was seen for the NALF CC16/urea ratio of girls (p=0.055). 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 (OR, 2.59, 95% CI: 1.15-5.82, P=0.02), as a ratio to urea, (OR, 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).

In both studies, defects in the nasal epithelium barrier function, 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. Levels of CC16 and albumin in the NALF of young children were suggestive of an immature epithelial barrier function. However, these results do not allow us to establish a cause/effect relationship between epithelial injury and allergic sensitization; therefore it seems to us necessary to carry out mechanistic studies that could explain what happens in the epithelium during the toxics and/or allergens exposure.

List of abbreviations

Alb	Albumin
APC	Antigen presenting cell
AR	Allergic rhinitis
BAL	Bronchoalveolar lavage
BMI	Body mass index
CC16	Clara cell protein
COPD	Chronic obstructive pulmonary disease
CPA	Cumulative pool attendance
CV	Coefficient of variation
EB	Evans blue
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
ELF	Epithelial lining fluid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
ICAM	Intercellular adhesion molecule
IFN-_	Interferon_
IgE	Immunoglobulin E
IL-_	Interleukin_
IQR	Interquartile range

MBP	Major basic protein
MC	Mast cell
MCP-4	Monocyte chemotactic protein-4
NALF	Nasal lavage fluid
NO	Nitric oxide
PAR-2	Protease-activated receptor-2
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RV	Rhinovirus
SCF	Stem cell factor
SD	Standard deviation
TARC	Thymus and activation-regulated chemokine
TGF-_	Transforming growth factor_
Th	T helper
TNF-_	Tumor necrosis factor_
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

1. Introduction

1.1 Allergic rhinitis

Allergic rhinitis is an IgE-mediated inflammation of the nasal mucosa, triggered by contact with certain substances foreign to the body called allergens. The symptoms are mainly represented by rhinorrea, congestion and sneezing. In their most severe forms, the local symptoms may be accompanied by other systemic ones, including malaise and fatigue. The allergic rhinitis can also occur in isolated form or be associated with other illnesses of allergic type, such as eczema, asthma and allergic conjunctivitis.

The allergens may be of a different type, and the period of exposure may be continuous or limited to a certain time of the year. For this reason, allergic rhinitis is classified into three broad categories: the perennial, seasonal and occupational rhinitis. The first is characterized by the persistence of symptoms in a more or less constant manner throughout the year, following exposure to allergens such as house dust mite, pet dander, molds, which are present in homes regardless of the season. In seasonal allergic rhinitis, symptoms appear instead in a specific period of the year, which is usually the time of pollination. In fact in this period there are in the air numerous pollens, which act as allergens. Once the pollination period is over, the symptoms disappear. Occupational rhinitis is however due to the combined exposure to irritants and allergens (Jefferey PK and Haahtela T, 2006; Quillen DM and Feller DB, 2006).

In short, the allergens, once in contact with the mucosa, cross the epithelial barrier and enter in contact with the antigen presenting cells (APCs), which process and present the allergens to Th2 lymphocytes. This is the initial phase of an allergic reaction, which takes the name of

sensitization. After the first contact with the allergen Th2, lymphocytes produce IL-4 and IL-13, which allow the activation of B lymphocytes and consequently the production of IgE. The presence of IgE at this point activates the cells that have receptors for IgE: the mast cells. These degranulate and release inflammatory mediators that are responsible for the onset of symptoms and the first phase of the allergic reaction called early phase response (Rombaux P et al, 2005). This appears during the first 10 minutes after the encounter with the antigen, and typically lasts 2-3 hours. The proinflammatory mediators, produced during the early phase response, act in such a way as to prolong the inflammation and thus give rise to the late phase response that appears 4-9 hours after antigen stimulation, and lasts between 18 and 24 hours (Jefferey PK and Haahtela T, 2006).

The cells mainly involved in allergic reactions are: lymphocytes, mast cells and eosinophils (Figure 1). The Th2 phenotype is characteristic of atopic subjects, and the activation of T helper 2 is a necessary condition for an allergic reaction can come about. In fact, the Th2 lymphocytes mainly produce IL-4 and IL-5 that stimulate, respectively, the IgE production by plasma cells and the activation of eosinophils (Mosmann TR and Sad S, 1996). In the case of an inflammatory response of the Th1 type cytokines, that are more present, are the IL-2 and the INF-γ, which counteract the action of IL-4 and therefore limit the effects of the allergic reaction (Erb KJ, 1999; Karmaus W and Botezan C, 2002). In the case of allergic rhinitis, the mucosa is intensely infiltrated by T lymphocytes (CD4 + and CD25 + T cells) and B cells (Pawankar RU et al, 1995). This is associated with a parallel increase in the number of IL-4, IL-5, IL-13 positive cells, which suggests that it is a reaction of Th2 type (Varga EM et al, 1999).

Even the mast cells (MCs) are part of the group of characteristic cells of allergic reactions, so as to be present in a significantly larger number in the mucosa of allergic subjects than in that of healthy subjects. Once activated, the mast cells degranulate and release numerous inflammatory

mediators, including histamine, prostaglandin D2, cysteinyl leukotrienes, and neutral proteases (Bousquet J et al, 2001), which, acting on the sensitive nerve endings, on the walls of blood vessels, and on the glands, are responsible for the itching, the nasal congestion, and the rhinorrhea (Jefferey PK and Haahtela T, 2006). The mast cells are divided into two groups: the MCs (T) that produce only tryptase, and MCs (TC), that in addition to tryptase, contain chymase, cathepsin G and carboxypeptidase. In subjects with allergic rhinitis, the MCs (T) are the predominant group. In addition to the most well-known substances, the mast cells produce a large number of mediators, such as IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF-_, which explains how these cells are not involved only in early phase response, but also modulate the inflammatory response in late phase response (Pawankar R et al, 2011).

To increase the action of the mast cells there are basophils, which are blood cells that under normal conditions are not encountered in the tissues, but which are abundant in allergic subjects. Their number is proportional to the severity of the disease. They produce histamine and cytokines like IL-4 and IL-13, but not Tryptase, and are involved in the late phase response (Pawankar R et al, 2011).

Eosinophils are typical cells of allergies, and have become the main object of mechanistic and therapeutic studies. These cells are of blood origin and are recruited *in situ* thanks to some chemokines, such as vascular cell adhesion molecule 1 (VCAM-1), RANTES, eotaxin, MCP-4 and Thymus- and activation regulated chemokine (TARC) (Lilly CM et al, 1997; Li L et al, 1999; Sekiya T et al, 2000) produced by epithelial cells, which also serve as chemoattractants for basophils and lymphocytes T. Once in situ, eosinophils begin to produce some of the most important mediators of the allergic reaction: MBP, ECP (Pawankar R et al, 2011), eosinophil-derived neurotoxin (EDN) (Broide DH et al, 1992), eosinophil peroxidase. Eosinophils also produce cytokines such as IL-3, IL-5, and GM-CSF, able to

prolong the survival of eosinophils in the mucosa for days or even weeks (Pawankar R et al, 2011).

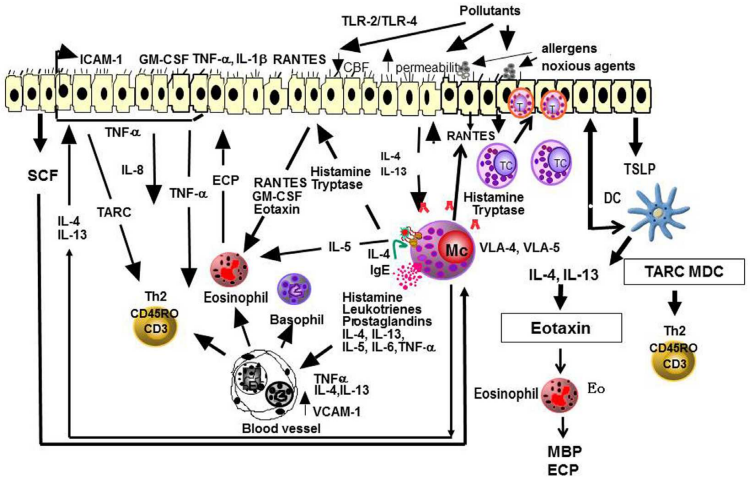


Figure 1: Ongoing inflammation in allergic rhinitis (from Pawankar R et al, 2002).

Subjects with allergic rhinitis experience many problems, related to both local and general symptoms of allergic rhinitis, which restrict patients in their daily activities. Often the difficulty in breathing makes it difficult to sleep, which results in daytime fatigue and has a negative effect on the performance both at school and work. These individuals are forced to avoid certain activities (sports, outdoor activities, etc.) which might expose them to allergens. In addition, some of them find annoying or embarrassing the need of having to blow their nose frequently. To this, the direct and indirect costs of the disease must be added. The direct costs are the costs of the treatment while the indirect costs relate to the reduced efficiency in the workplace. For these reasons, allergic rhinitis has become more and more an object of study and research, particularly in industrialized countries, where cases have increased in recent years (Camelo-Nunes IC and Solé D, 2010).

1.2 Epidemiology

The history of allergic rhinitis begins in 1819, when Bostock described for the first time his "periodical affection of the eyes and chest," to which he gave the name of "summer catarrh" (Garrelds IM et al, 1996). Currently, the prevalence of allergic rhinitis ranges from 9% to 42% in the general population worldwide (Tang XF et al, 2012), and in the infant population, values oscillate between 20% and 40% (Hardjojo A et al, 2011; Penaranda A et al, 2012; Nahhas M et al, 2012; Siriaksorn S et al, 2011; Castro LK, et al, 2010). Over the last 30-40 years, the prevalence of allergic diseases, including allergic rhinitis, has continued to increase, so that in industrialized countries 25% of childhood diseases are allergic (Torres-Borrego J et al, 2008).

Most of the information available to us comes from studies conducted in Western countries, mainly in Europe and in the US, where the prevalence of allergic rhinitis ranges between 10 and 30%. Data for developing countries are less consistent probably because of a lack of information in the public domain and changes in socio-economic situation. In China and in the Middle East, the prevalence of allergic rhinitis seems to have increased most likely because of an increasing exposure to those factors linked to Western lifestyle that promote the development of allergies (figure 2) (Katelaris CH et al, 2012). In Europe, the prevalence of rhinitis is usually higher in the Northern than in Southern countries: 28.5% in Belgium (Mullol J et al, 2008), 26.0% in UK (Bauchau V and Durham SR, 2004), 24,5% in France (Bauchau V and Durham SR, 2004), 21.5% in Spain (Sánchez-Lerma B et al, 2009), 20.6% in Germany, 16.9% in Italy (Bauchau V and Durham SR, 2004), 5.2% in Cyprus (Kolokotroni O et al, 2011).

This ’allergic epidemic‘ appears to involve people who do not necessarily have a history of atopy in the family, which has led scientists to

direct their attention away from a possible genetic predisposition, towards a change in the surrounding environment and consequently exposure to certain ’environmental factors’.

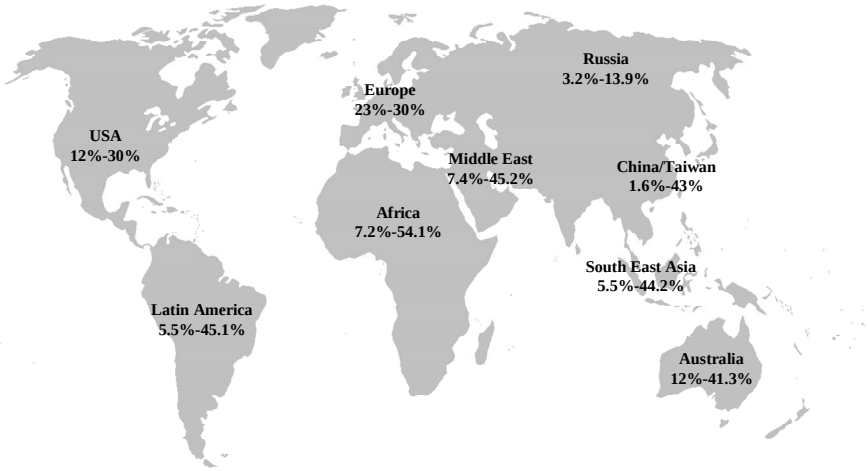


Figure 2: Prevalence of AR or AR/C in different regions of the world (adapted from Katelaris CH et al, 2012).

1.3 Main risk/protective factors

1.3.1 Inhalant factors

1.3.1.1 Air pollution/ endotoxins

Several studies report a higher prevalence of allergies in the city than in the countryside (Trigg CJ and Davies RJ, 1991; Alm B et al, 2011). In particular, it seems that children who have parents who are farmers exhibit lower rates of sneezing attacks, allergic sensitization, wheezing, itchy skin, and allergic rhinitis (Braun-Fahrlander C et al, 1999; Von Ehrenstein OS et al, 2000 ; Riedler J et al, 2000). Moreover, adults who grew up in a farm environment have a reduced risk of allergic rhinitis (Karmaus W and Botezan C, 2002). These results are even more evident in organic farmers (Smit LA et al, 2007). No definite cause has been established; two hypotheses have been formulated: one that investigates the cause in the air pollution of the cities, the second in the exposure to endotoxin in the farm environment.

The impact of air pollutants (diesel exhaust, ozone, etc.) on the airways can be summarized as: irritation, inflammation, bronchoconstriction and sensitization. A pollutant can be responsible for more than one of these effects, the severity of which depends on the concentration of the chemical in the environment, and the health condition of each individual (Karol MH, 1991). Ultra-fine particulate matters can be deposited in the intercellular spaces and in transudate between epithelial cells, causing a transformation of the normal nasal epithelium in a squamous metaplastic epithelium. This results in a reduced ability to filter out inhaled gases and particulate matters, which remain therefore in contact with the nasal mucosa a period of time longer than normal (Garciduenas Calderon L et al, 2007). But the main

mechanism by which the pollutants facilitate the allergic response is the absorption of aeroallergens, which causes a longer permanence and concentration of these in the atmosphere and consequently a greater risk of exposure. In addition, the tissue damage caused by pollutants facilitates the absorption of allergens (Torres-Borrego J et al, 2008).

According to the second hypothesis, the cause does not lie in exposure to pollutants, but rather in exposure to endotoxins, more prevalent in a farm environment rather than in the city. The endotoxins stimulate an immune response of Th1 type, at the expense of that of Th2, notoriously associated with allergic reactions. However, recent studies have questioned the latter hypothesis, showing an increase in respiratory problems in people living in the countryside (Karmaus W and Botezan C, 2002; Vogelzang PF et al, 2000; Sprince NL et al, 2000; Douwes J et al, 2000).

1.3.1.2 Allergens

Some studies emphasize the protective aspect of exposure to pets (R de Marco et al, 2004; M Waser et al, 2005; B Holscher B et al, 2002). This protection might be result of a bias related to pet avoidance because of allergic diseases in the family. In any case, the age of first exposure (for example, the window of immune maturation) is fundamental for the development of allergies (Tanaka K et al, 2007).

Dampness in the home is linked with an increased risk of allergic rhinitis (Tamay Z et al, 2007; Kuyucu S et al, 2006; Biagini JM et al, 2006; Stark PC et al, 2005; Chen WYet al, 2003; Stazi MA et al, 2002): in fact, it promotes the development of dust mites, fungi and bacteria and increases the number of cockroaches (Jaakkola MS et al, 2006). In addition, moisture may come from chemical emissions from building materials, which, as irritants, may indirectly promote allergies.

1.3.1.3 Smoking

Numerous studies have shown that smoking is a risk factor in the development of allergic diseases. The issue of passive smoking is less clear-cut: the results are contradictory. In Trinidad and Tobago, where the temperate climate allows children to play outside (and are therefore less exposed to passive smoke), Monteil (Monteil MA et al, 2004) found a strong correlation between passive smoking exposure and the prevalence of asthma and rhinitis. Another study conducted in Sweden suggests the opposite: a lower risk of rhinitis in children exposed to secondhand smoke (Hjern A et al, 2001). Contradictions were also found with regard to maternal smoking during pregnancy: half of the studies did not confirm an influence on the risk for children to develop atopic disorders. (Tanaka K et al, 2007). These results can put down to bias, mainly due to the inability to quantify the exposure from maternal smoking, and then to a lack of control for potential confounders in most of the published analyzes, such as the potential of the bias towards an inverse association if parents of atopic children avoid smoking (Strachan DP and Cook DG, 1998).

1.3.1.4 Swimming pool

Chlorination is the name given to the process of purification effected through the use of chlorine. The first time that chlorine was used to disinfect the water at the beginning of the last century, when chlorine allowed making drinking water safe. Chlorine has been adopted for water disinfection in swimming pools more recently, mainly in the form of hypochlorite, chlorine gas or chloroisocyanurates. These products once in the water release hypoclorous acid, a non selective biocide that oxidizes all forms of organic matter, including that brought by swimmers, such as sweat, tears, urine, ect.,

releasing a mixture of harmful byproducts, including such irritants as chloramines and haloacetic acids (Figure 3) (Kim H et al, 2002; Bernard A, 2007).

Among the chloramines, the mono- and di-chloramine remain in the water or on the surface in the form of aerosols, while the highly volatile trichloramine is released in the air of indoor swimming pools, giving them their characteristic "chlorine smell". The irritant action of these substances has long been underestimated or otherwise considered insignificant

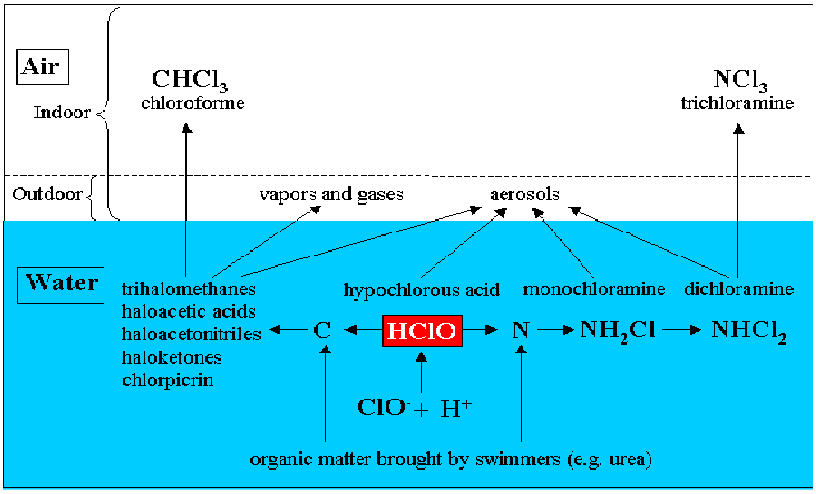


Figure 3: Main chlorination products in the water and air of indoor and outdoor pools (adapted from Voisin C and Bernard A, 2008).

compared to the advantage of ensuring water disinfection.

Only recently studies have been conducted to examine the potential effects swimming pool water chlorination on the health of swimmers. In fact, a higher incidence of asthma was observed in subjects who practice swimming (Bernard A, 2007). This result was considered to be a selection bias due to the fact that swimming is a sport recommended for asthmatics

because of its low asthmagenecity, and therefore the population of swimmers has a higher rate of asthma than other sports. However, this explanation does not hold for cases of allergic rhinitis, or in association with some respiratory symptoms (cough and shortness of breath) in the absence of a diagnosis of asthma. Moreover, these associations may not be attributable to the simple sporting activity, since they are not found in swimmers of copper-silver pools (Bernard A et al, 2009). The hypothesis of the irritant effect of chlorine on the nasal mucosa is accredited by the results of a recent study carried out on a population of swimmers with allergic and non-allergic rhinitis (Gelardi M et al, 2012). In this study, the use of a nose clip for 30 days caused a symptomatic improvement in all participants and a reduction of the nasal resistance and of the neutrophilic component of inflammation in subjects with neutrophilic rhinitis. According to some authors the attendance of chlorinated pools is *per se* not sufficient to cause the development of an allergic disease but can exert an adjuvant effect by interacting with the atopic status. Helenius I et al. (1998) showed that the risk of asthma was 10 times higher among the competitive swimmers who were atopic than in those who were not. Nystad W et al. (2003) also observed a higher prevalence of wheezing among swimming children who had atopic parents than those without.

To explain these results, it was hypothesized that the chlorination byproducts are able to destroy the intercellular junctions and thus promote the penetration of allergens across the epithelial barrier and their interaction with cells of the immune system, using a mechanism similar to that of allergens presenting a proteolytic activity (Figure 4).

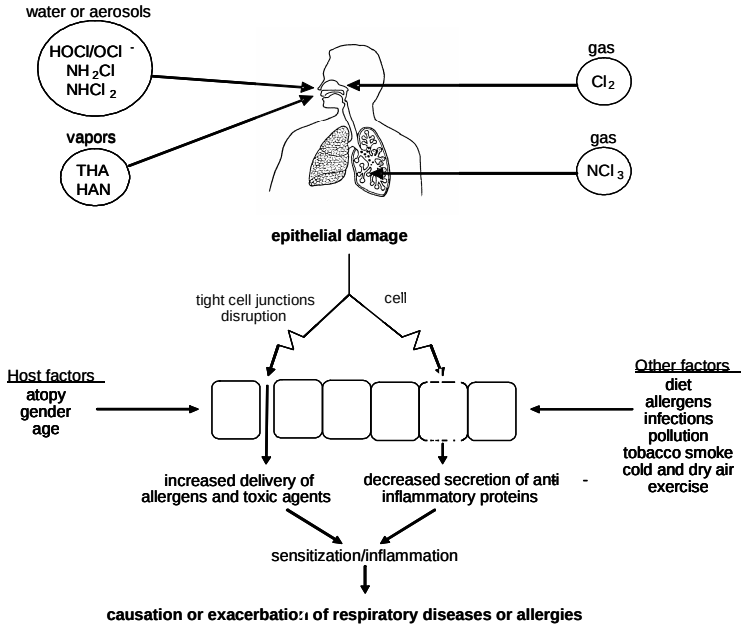


Figure 4 : Effects of chlorination by products on the respiratory epithelium.

1.3.2 Non-inhalant factors

1.3.2.1 Birth period

It has been speculated that the time of birth may have an influence on the development of allergic rhinitis, the consensus being that the spring was the time of greatest risk, because it is the most important pollination period of the year, and the period of greatest risk of exposure to aeroallergens. Disparate studies have been conducted on the subject, obtaining results that confirm the initial hypothesis: children born in spring in Scandinavia have an increased risk of birch pollen rhinitis (Biorksten F et al, 1991). In Tasmanian

children born during the pollen season, early life viral upper respiratory tract infections were found to interact with ryegrass allergen exposure to promote the ryegrass allergen sensitization (Kemp A et al, 2009).

However, Sibbald and Rink (1990) observed that the subjects most at risk were those born in the summer, and concluded that this result was due to winter respiratory tract infections at a time when transferred maternal immunoglobulin concentrations have fallen below protective levels.

1.3.2.2 Breastfeeding

The effect of breastfeeding on the development of allergies remains controversial, some studies report a positive correlation, others no correlation, others a negative correlation (Gdalevich M et al, 2001 ; Wright A et al, 2001; Obihara CC et al, 2005). The inconsistency of these results is probably derived from a bias: in a family with an atopic history, mothers have a tendency to prolong the breastfeeding that may mistakenly be as a predisposing factor for the development of allergic diseases. (Torres-Borrego J et al, 2008).

1.3.2.3 Diet

1.3.2.3.1 Antioxidants and fats

The intake of antioxidants seems to be linked to a reduction in the prevalence of asthma; on the contrary, a diet rich in monounsaturated fatty acids is associated with a higher risk (Trak-Fellermeier MA et al, 2004).

However, these results may be biased by other protective factors that are associated with healthy eating habits, such as a higher socioeconomic level, a longer duration of breastfeeding or a less exposure to tobacco smoke,

among others. (Torres-Borrego J et al, 2008).

1.3.2.3.2 Fish

The risk of allergic rhinitis in children appears to decrease when they were eating fish in their earliest months of life; it is not so much the frequency of intake that has an influence on the emergence of allergies, but rather the precocity of ingestion. Probably the intake of omega-3 in fish has implications on the development of the immune system and the Th1/Th2 balance, although the possibility of the action of other components of the fish cannot be excluded (Alm B et al, 2011).

1.3.2.3.3 Probiotics

It has been demonstrated that the intestinal flora plays an important role in Th1/Th2 balance. In fact, there are differences in the composition of intestinal microflora, between allergic and non-allergic subjects: the former has a prevalence of Clostridium difficile and coliform species and S. aureus, while the latter of Lactobacillus. Kimoto et al., (2004), studied the immune modulatory activity of 15 strains of Lactobacillus on murine macrophages: 6 strains induced the production of IL-2, IL-6 and TNF-α. Among these 6 strains, there is one (the G50 strain) that induces more cytokine production. Although destroyed by heat, G50 continues to stimulate the production of cytokines, which suggests that this activity is linked to some elements of the bacterial wall. (Rodrigues Nogueira JC and da Conceição Rodrigues Gonçalves M, 2009).

In recent years, numerous studies have been performed to analyze the influence of the intake of probiotics on the emergence of allergic diseases, even if there still remains the problem of the choice of probiotics, not knowing the exact composition of the intestinal microflora in healthy

subjects (J Torres-Borrego et al, 2008).

Probiotics are supplements of microorganisms whose existence has known for 100 years: the first, who discovered them, was Metchnikoff that found a higher life expectancy among the Bulgarian villagers who drank fermented milk. The main probiotics are: *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus paracasei, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium adolescentis, Saccharomyces bourlardii, Propionibacterium freudenreichii;* and also *Escherichia, Enterococcus* and *Bacillus* and the *Saccaromyces boulardii* fungus. By definition, a probiotic must comply with specific criteria: it must be a natural dweller of the gastrointestinal system, must be resistant to gastric juice and the bile, must have a precise taxonomic definition, be able to colonize the intestinal mucosa and be active before being ingested (JC Rodrigues Nogueira da Conceicao Rodrigues and Goncalves M, 2009).

Giovannini et al. (2007) in a randomized study (pre-school children with asthma and/or AR, who received either placebo or Lactobacillus casei for 12 months) discovered a reduction in allergic episodes during the year, and an increase in the length of asthma- and AR free periods. In 2007, Odamaki et al. observed in allergic individuals, during the period of pollinosis, a change in the intestinal microflora, which was reduced by the intake of probiotics. This microbiota fluctation was most evident at the beginning and at the end of pollinosis, perhaps due to the stress caused by symptoms of AR; no valid scientific explanation has been found.

1.3.2.4 Gender

It is known that prepubescent males exhibit a higher prevalence of allergic sensitization, rhinitis and asthma (Sears MR et al, 1993; Mensinga T et al, 1994; Torres-Borrego J et al, 2008) (with the exception of atopic dermatitis, which is more prevalent among females at any age). This trend is reversed during adolescence (Shamssain MH and Shamsian N, 2001) and seems to be due to the effect of estrogen hormones, which are proinflammatory, unlike the steroids that are immunosuppressants (Torres-Borrego J et al, 2008). Navarrete-Palacios E et al. (2003) observed that the various phases of the menstrual cycle were associated with the cytological changes at the level of the nasal mucosa, which corresponded to similar changes at the level of vaginal mucosa, suggesting that cell turnover in the nasal epithelium is influenced by hormonal state. Moreover, women seem to have smaller nasal dimensions than men (Walinder R et al., 2000).

1.3.2.5 Infections

Strachan formulated in 1989 the hygiene hypothesis, according to which the increasing incidence of allergic diseases in the western world must be sought in a reduction in exposure to common infectious agents. According to this theory, frequent infections lead to an increased production of IL-12, IL-18 and IFN-_, all cytokines that inhibit the Th2 response, responsible for allergic reactions. A study on 24,341 mother-offspring couples has observed that the predisposition to develop allergic diseases is not so much associated with infections in infancy, as it is with a number of factors associated with exposure to pathogens (such as number of siblings, nursery attendance, living on farms or having pets in the home), which would seem to have a protective effect. These results suggest that the concept of "microbial

burden" is more important than the existence of specific infections as a protective factor in childhood (Torres-Borrego J et al, 2008).

1.3.2.6 Siblings

The phenomenon known as the "siblings' effect", described for the first time by Golding and Peters (1986), derived from a series of studies carried out with the aim of assessing whether the fact of having older siblings could have an influence on the development of diseases. As a result, asthma and allergic rhinitis are negatively correlated with the number of siblings, a correlation that becomes even stronger with regard to older siblings. (Karmaus W and Botezan C, 2002). For each pregnancy, a mother's increased tolerance to allergens and a reduction in the levels of IgE in umbilical cord were also observed (Torres-Borrego J et al, 2008; Karmaus W et al, 2004; Karmaus W et al, 2001). However, the mechanism that underlies this phenomenon still remains unknown.

1.3.2.7 Vaccinations

Even this is a controversial subject, for some, the systematic vaccination, especially against whooping cough and measles, is responsible on the one hand of a lower susceptibility to infections and therefore of a reduction of the protective effect of the latter and on the other hand of an increased production of IgE (Farooqi IS and Hopkin JM, 1998; Kemp T et al, 1997). However, other studies do not confirm these data and do not observe any correlation between vaccination and allergic diseases (Bernsen R et al, 2005). According to Anderson et al. (1995), this is the result of the different vaccination methods used in different countries (Torres-Borrego J et al, 2008).

1.4 Epithelium

The nose is a very important organ in the protection of the distal airways from harmful pathogens and toxic substances. Everyday about 12,000 liters of air pass through the nose where they are hydrated and filtered.

The nasal cavity is carpeted with 120 cm2 of a respiratory mucosa having a thickness between the 0.3 and 5 mm. The lining epithelium is a pseudostratified columnar ciliated epithelium formed by different cell types. The basal cells rest on the basement membrane and reach the surface, while the columnar cells extend for the whole height of the epithelium. On the pole apical, numerous cilia can be observed with a 6 μm lenght and a width around 200 nm (Widdicombe JH et al, 1997). On the inside, they are crossed by 9 double tubules around two central tubules. Interposed between the ciliated cells, there are goblet cells and intraepithelial mucous glands, involved in the production of nasal mucus.

The epithelium rests on a basement membrane thick 1.6-10 μm below which there is a connective tissue rich in capillaries and glands. Among these the majority is represented by seromucous glands, to which the serous glands and the mucous glands must be included (Beule AG, 2010).

One of the main functions of the nose is to clean the air entering the airways. The nose is capable of removing 95% of particles above 15 μm, while for those of lower size effectiveness is reduced (Beule AG, 2010). The mucus is formed by a network of linear, hydrated mucin molecules capable of forming unspecific secondary connections, e.g. with pathogens or drugs. Furthermore, the protective action against infection is increased by the slight acidity of the nasal mucus, where the PH is around 5.5 and 6.5. The mucus is produced mainly by glands, the goblet cells, the transudation of blood plasma, mucosal tissue fluid and tear fluid (Beule AG, 2010). In the the mucus cilia beat in synchronous at a speed of 2-25 mm / min. The

mechanism that controls the speed of the beating of cilia is not yet clear, but some substances such as the NO or the IL-13 can influence the frequency of the beats (Laoukili J et al, 2001; Beule AG, 2010).

Once damaged, the epithelium may need a more or less long period to reform depending on whether the basement membrane is intact or not: in the first case the restoration is rapid, in the second it can take weeks or even months (Beule AG, 2010). The nostrils are subject to cyclical changes of the state of the mucosa congestion, a phenomenon known as the nasal cycle. The nasal cycle is regulated by the hypothalamus and the two phases of the cycle (working and resting) are easily recognizable: in the working phase there is an increase of the hydraulic diameter and of the passage of airflow and turbulence, while the resistance decreases. The opposite occurs during the resting phase. Even the mucociliary clearance is 2.5 times greater in the nostril working that in the resting (Lang C et al, 2003; Beule AG, 2010).

The proper functioning of the mucociliary apparatus and the epithelial integrity provide a mechanical barrier that limits the encounter between defense cells and allergens. The epithelial integrity can be ensured only by the integrity of tight junctions. These are macromolecular assemblies of proteins that form continuous rings at the apices of epithelial cell. The tight junctions proteins ZO-1, ZO-2, ZO-3, AF-7, symplekin, 7H6 and cingulin are located within the cytoplasm (Anderson JM and Van Itallie CM, 1995; Haskins J et al, 1998; Denker BM and Nigam SK, 1998), while occludin and claudins are transmembrane proteins (Furuse M et al, 1993; Ando-Akatsuka Y et al, 1996; Furuse M et al, 1994; McCarthy KM et al, 1996; Furuse M et al, 1998). External perturbing factors can destroy the tight junctions and thereby alter epithelial permeability. Among these factors there are some allergens having a proteolytic activity, such as house dust mite (HDM). The HDM allergen Derp1 can act enzymatically breaking and making displace the tight junctions from their normal apical position on the desmosomes

(Farquhar M and Palade GE, 1963).

The mechanism of action has long been discussed: the Derp1 acts directly on the tight junctions or activates a cell surface zymogen that then degrades occludin. Wan H. et al., in 2000, found the first hypothesis the most realistic, taking into account the action, inhibiting the degradation, of occludin itself and the ineffectiveness of serine, aspartic and metalloproteinase inhibitors to block the degradation of occludin by the Derp1. However, several studies have shown that the HDM allergens Derp1, Derp3 and Derp9 are able to activate PAR-2 (protease-activated receptor-2) in lung epithelial cells (Sun G et al, 2001; Asokananthan N et al, 2002). Cockroach allergens stimulate PAR-2 in mouse lung fibroblasts and in human respiratory epithelial cells (S Kondo et al, 2004; JH Hong et al, 2004; Jeong SK et al, 2008).

The injury leaves the junctional epithelium exposed to the harmful action of toxic agents and infectious environmental factors that aggravate the wound tissue and slow the repair process (Herard AL et al, 1996). The increased sensitivity to the action of external factors has been demonstrated by the fact that the cultured cells from asthmatic subjects have a reduction of transepithelial resistance at much lower concentrations of smoke extract than that necessary to obtain the same results in normal epithelium (Holgate ST, 2008).

However, it is a mistake to consider the nasal epithelium only as a mechanical barrier against external agents, recently it was discovered that the role of the epithelial cells in allergic reactions is much more complex. First, epithelial cells are capable of producing inflammatory agents, such as eicosanoids, endopeptidases, cytokines and chemokines (IL-6, IL-8, GM-CSF, TNF-α, RANTES, TARC, eotaxin, SCF), release the matrix metalloproteinases 2,9,13 and express adesion molecules as ICAM-1 (Pawankar R et al, 2004). They can also act as antigen-

presenting cells to T cells. Some studies have shown that HDM allergens can activate the epithelial cells in vitro, without the intervention of an IgE mediated reaction (Thompson PJ, 1998, King C et al, 1998). Furthermore epithelial cells produce cell-derived thymic stromal lymphopoietin (TSLP), IL-25, IL-33 that amplify the Th2-type allergic inflammation (Wang YH et al, 2007; Préfontaine D et al, 2009). Even the endothelium participates actively in the allergic response: it, as the nasal epithelium, presents H1-receptor for histamine through which it is activated. In addition, they are actively involved in the recruitment of inflammatory cells, overexpressing VCAM-1 and producing various cytokines and chemokines such as RANTES and eotaxin (Pawankar R et al, 2011).

Remodeling may be defined as the process of rebuilding following a tissular lesion, which may result in a complete tissue repair or in a replacement of the healthy tissue with a pathological one. Despite the fact that inflammation between upper and lower airways is very similar, the same can not be said for the remodeling, which appears to be less pronounced in the nasal mucosa. In fact, when the asthma makes its first appearance, on the mucosa phenomenon of the so-called remodeling can be observed. The remodeling is a structural change of the tissue which consists of an infiltration of macrophages and lymphocytes, the angiogenesis, the thickening of the basal lamina and the epithelial fibrosis. The latter is due to the proliferation of fibroblasts, and subepithelial myofibroblasts that produce new matrix including tenasin C, fibronectin and typers I, III and V collagens. Recent studies have shown the ability of some epithelial cells to differentiate into fibroblasts under the action of some profibrotic cytokines such as TGF-β (Leung SY et al, 2006; Locke NR et al, 2007; Holgate ST et al, 2008).

At the nasal level apparent structural changes are not observed, probably because of the absence of an intense mechanical traumatic event such as bronchoconstriction in the lungs, or also because of the different patterns of remodeling due to the different embryological origins of the two

organs (ectodermal for the nose, endodermal for the lung). The most important changes in the mucosa are observable through the use of electron microscopy: cytoplasmic vacuoles and increased intercellular space. Ciliated cells dysplasia or metaplasia can be found (Gluck U and Gebbers J, 2000). Similarly to asthma, also in allergic rhinitis, a thickening of the reticular portion of the basement membrane and a deposit of I and III collagens can be observed. Perhaps the thickening of the basal lamina helps stabilize the epithelium mechanically, as well as collagen deposits to avoid excessive expansion of the nasal mucosa, thus keeping the nasal cavity patent. Other alterations are a compression, atrophy and dilation of the glands in the submucosa, while the phenomenon of angiogenesis is confirmed by the presence of high levels of angiogenic factors such as platelet derived endothelial cell growth factor and the VEGF in patients with rhinitis (Salib RJ and Howarth PH, 2003; Bousquet J et al, 2004; Constantino G de T and Mello Jr JF, 2009).

1.5 Biomarkers

1.5.1 Clara cell protein (CC16)

The Clara cell protein (CC16) is a small protein (16kDa) produced by non-ciliated cells of the distal airways. The function of this molecule has not yet been completely elucidated; however, it has been demonstrated that the CC16 is able to inhibit the activity of numerous components of the inflammatory response, such as phospholipase A2, INF-_, TNF-_, as well as platelet derived growth factor and modulate Th2 responses (Dierynck I et al, 1995; Ramsay PL et al, 2003; Hung CH et al, 2004; de Burbure C at al, 2007; Johansson S et al, 2007). The CC16 in bronchoalveolar lavage (BAL), serum and sputum, has been used as biomarkers of epithelial integrity in

pulmonary disease in numerous states, such as: fibrosis, sarcoidosis, COPD, asthma, occupational and environmental lung injury (Ye Q et al, 2004; Hermans C et al, 2001; Gioldassi XM et al, 2004; Palczynski C et al, 2005; Shijubo Net al, 1999; Blomberg A et al, 2003, Michel O et al, 2005; Pilette C et al, 2001).

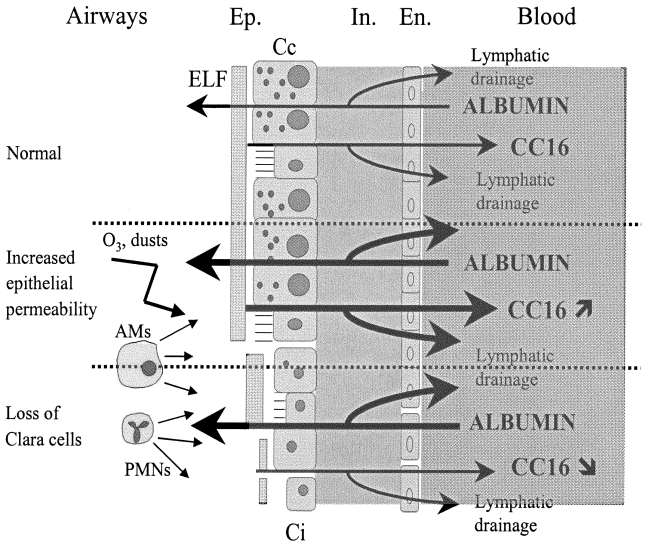


Figure 5: Schematic representation of the passage of albumin and CC16 through the epithelial barrier. En., endothelium;Ep., epithelium;In., interstitium;Cc, Clara cell;Ci, ciliated cells;ELF, epithelial lining fluid.

A decrease of CC16 in the airways is due to: a destruction of the Clara cell, an increase in the permeability of the epithelial barrier with consequent leakage of protein, changes in transcriptional activity within remaining Clara cells.

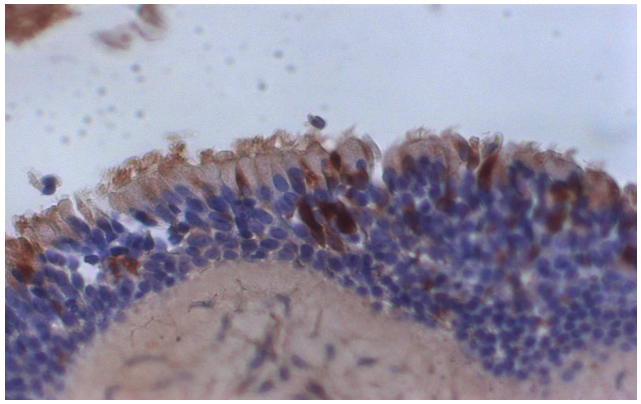


Figure 6: CC16 immunostaining in human nasal biopsy (Sardella A et al, unpublished).

In mice, lung injury is characterized by a dedifferentiation of epithelial cells and an alteration of the transcriptional activity of ciliated and non-ciliated cells (Park KS et al, 2006; Kropski JA et al, 2009; McAuley DF and Matthay MA, 2009). In all these cases a reduction of the concentration of CC16 in BAL is observed, but, only in case of an increase in epithelial permeability, this is accompanied by an increase of CC16 in the blood (Figure 5) (Determann RM et al, 2009; McAuley DF et al, 2009; Broeckaert F et al, 2000; Bernard et al, 1994).

CC16 were found in subjects with allergic rhinitis and nasal polyps compared to healthy subjects (Benson M et al, 2007; Lensmar C et al, 2000; Lindahl M et al, 1999; Johansson S et al, 2005). But the most interesting discovery regarding nasal CC16 was made by Benson M. et al., who performing an immunostaining on the nasal biopsies of allergic subjects, discovered that CC16 is produced by the cells of the nasal epithelium, which does not contain Clara cells (Figure 6).

1.5.2 Albumin

Albumin is a plasma protein produced by hepatocytes. Albumin present in the nasal cavity comes from the blood, and its increase reflects an alteration of epithelial permeability. This is why it has been used in several studies as biomarkers to quantify the epithelial damage upon exposure to infectious agents or irritants or to monitor the action of various drugs. The albumin is higher in nasal lavage of patients with allergic rhinitis compared to healthy subjects, but tends to decrease after administration of fluticasone propionate (Schaper C et al, 2010), fexofenadine (Shaper C et al, 2009) and omalizumab (Hanf G et al, 2004). Probably this is due to the inhibitory effect of these medicines on mediators of allergy such as histamine, kinins, leukotrienes or cytokines, which contribute to the vascular hyperpermeability.

1.5.3 Urea

Urea concentrations in NALF are usually employed for estimation of the nasal epithelial lining fluid and dilution of NALF (Semple MG et al, 2007; Cavaliere F, 1986). A study managed by Kaulbach H.C. et al. has confirmed that, after the collection of undiluted nasal secretions, plasma urea and nasal urea concentrations are equivalent even after glandular stimulation or induction of vascular permeability. One can reasonably consider the nasal urea to plasma urea ratio as a dilution factor to adjust biomarker concentrations in NALF.

2. Objectives

The goal of this project is to develop a system for monitoring the health and the integrity of the nasal epithelium, through the measurement of non-invasive biomarkers, and to use it to assess the impact of environmental risk factors on the nasal mucosa. The second aim of the project is to find out when the epithelial lesions appear for the first time, and since the age of onset and the severity of damage can act as predisposing factors for allergic sensitization.

The technique, that we have chosen, is the nasal lavage, which is a non-invasive method that allows the recovering of a portion of the proteins contained in the nose. Among these, we decided to analyze the CC16, albumin and urea. CC16 was chosen because it reflects the damage to the epithelial cells, albumin was chosen as it is an index of epithelial permeability, and urea because it allows correcting any bias due to sample dilution.

The first population is represented by 474 adolescents aged between 15 and 18 years, from three different schools in Belgium: Louvain-la-Neuve, Lessine and Bastogne. Parents received a questionnaire to fill out, with questions on health status, on allergic diseases, on use of medicines, on family history and on exposure to risk factors for their children. The volunteers were subjected to a nasal lavage, in which the concentrations of biomarkers were analyzed.

In the second study, the cohort is made up of 288 children between 4 and 6 years, from different schools in the south of Belgium. The same protocol as in the first study was applied to this population.

The purposes of the first study are to test the applicability of the nasal lavage as non-invasive test in studies involving a large number of participants and further to analyze the correlations between epithelial

lesions, exposure to environmental stressors and onset of allergic sensitization.

The goals of the second study are to find out whether the epithelial lesions appear already in a very young age and to study associations between tissular damage, environmental risk factors and development of allergic sensitization. At the same time we want to analyze if the immaturity of the nasal epithelium may explain its susceptibility to allergic sensitization at a very young age.

3. Results

3.1 Study 1: Nasal epithelium integrity, environmental stressors, and allergic sensitization: a biomarker study in adolescents.

3.2 Study 2: Nasal epithelium biomarkers in young children: Associations with allergic sensitization and environmental stressors.

3.1 Study 1: Nasal epithelium integrity, environmental stressors, and allergic sensitization: a biomarker study in adolescents.

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Conception and design: AB, AS, IAM; Examination of adolescents and biological analyses: AS, MN, CV, MN, XD, AB; Data analysis and interpretation: AS, AB; Drafting the manuscript: AS, AB. AB is the guarantor of the paper and takes responsibility for the integrity of the work as a whole, from inception to published article.

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Running title: nasal epithelium biomarkers

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

Abbreviations: BMI, body mass index; CV, coefficient of variation, NALF, nasal lavage fluid; CC16, Clara cell protein; CPA, cumulative pool attendance; ELF, epithelial lining fluid; IQR, interquartile range; SD, standard deviation

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ABSTRACT

Changes in the airways epithelium caused by environmental insults might play a role in the development of allergic rhinitis. We measured albumin and Clara cell protein (CC16) in the nasal lavage fluid (NALF) from 474 adolescents (263 girls). The NALF CC16/albumin ratio, integrating the permeability and cellular integrity of the nasal epithelium decreased mostly with time spent in chlorinated pools. In boys, a lower CC16/albumin ratio in NALF was associated with an increased risk of house dust mite sensitization. The results suggest that the CC16/albumin ratio in NALF can be used to detect nasal epithelium alterations linked to allergic sensitization.

INTRODUCTION

Allergic rhinitis and atopic asthma are chronic inflammatory diseases of the airways that develop following sensitization to airborne allergens and progressively lead to an impairment of lung function. Patients with allergic asthma frequently also suffer from an allergic rhinitis, which frequently precedes the onset of asthma. Both diseases also share the same genetic traits and the same triggers including allergen exposure, viral infection and air pollution, which suggests that they might be the manifestations of the same underlying allergic disease (Marple 2010; Spergel 2010; Murphy and O'Byrne 2010). Although allergic diseases are driven by the immune system, in particularly by the activation of Th2-type lymphocytes, the idea now progressively emerges that the primary cause of these diseases might not lie in the immune processes but in some functional or structural defects of the airways epithelium (Cookson 2004; Holgate et al, 2009). When occurring in certain windows of exposure or susceptibility, in particular during early life, these airways epithelium defects might create conditions promoting the

allergic sensitization and later the transformation of the atopic phenotype into rhinitis and then asthma (Holgate et al, 2009). Changes in airways epithelium, which might predispose to allergic diseases are largely unknown. One possible type of change might be an epithelial hyperpermeability facilitating the transepithelial delivery of allergens to the antigen-presenting cells (Bernard et al, 2003). This mechanism is suggested by the fact that most potent allergens such as the house dust mite allergens present a proteolytic activity enabling them to open the tight junctions and thus to move more easily across epithelial barriers towards immunocompetent cells (Cho et al, 2010; Roelandt et al, 2008). Ambient air pollutants or lifestyle-related stressors with a membrane disrupting potential such as ozone, tobacco smoke, chlorination products or endotoxins are also capable of opening tight junctions and thus of exerting a similar adjuvant effect in allergic sensitization (Carbonnelle et al, 2002; Bernard et al, 2003; Schelegle et al, 2003; Michel et al, 2005; Chiminti et al, 2010). Another possible mechanism which might be triggered by above stressors would be a damage or dysfunction of epithelial cells secreting proteins downregulating the allergic or inflammatory responses. The reduced secretion of the anti-inflammatory Clara cell protein (CC16, SCGB1A1 or secretoglobin 1A1) (Jackson et al, 2011; Miele et al, 1987) in subjects exposed to chlorination products (Lagerkvist et al, 2004; Bernard et al, 2007) and in patients with asthma (Laing et al, 2000) or allergic rhinitis (Benson et al, 2007; Johansson et al, 2005) might be illustrative of such a mechanism. Since cell damage in the airways is frequently accompanied by an increased epithelial permeability, the pulmonary pool of anti-inflammatory proteins might be further reduced by the leakage of lung proteins across the disrupted bronchoalveolar blood barriers (Hermans and Bernard, 1999).

The purpose of this population-based study conducted among school adolescents was to test these different hypotheses by assessing the

associations between nasal epithelium integrity, possible environmental insults and the risks of allergic sensitization. We evaluated the integrity of the nasal epithelium by measuring in nasal lavage fluid (NALF) albumin as a marker of epithelial permeability and CC16 as a marker of cell damage or dysfunction.

MATERIALS AND METHODS

Population Studied

After approval of the study by the Ethics Committee of the Faculty of Medicine of the Catholic University of Louvain (no IRB number), a total of 870 adolescents, aged between 15 and 18 years were recruited from three secondary schools in Belgium. The study protocol was described in detail previously (Bernard et al, 2008). In order to avoid acute effects caused by irritants or inflammation, we excluded subjects who had signs of rhinitis or who were under medication at the time of examination as well as those with insufficient NALF volume to perform all analyzes. The final population retained for the study included 263 girls and 211 boys.

Nasal lavage sample collection analysis

NALF samples were obtained from both nostrils using a procedure similar to that described by Tossa et al. (2009). Briefly, participants were asked to sit down, bend forward and put their heads down. Two and a half ml of sterile physiological water at 37°C were instilled into each nostril by a disposable tip connected to a peristaltic pump. After 10 seconds, students were asked to lift their head and the NALF was collected using a small funnel. Participants were free to start the NALF collection with the left or

right nostril. As the majority of participants were right-handed, most of them started with the right nostril. CC16 was quantified by a semi-automated non-isotopic immunoassay based on the agglutination of latex particles coated with a polyclonal antibody (Bernard et al. 1992a,b). We used a rabbit anti-human CC16 polyclonal antibody (A0257 purchased from DAKO) and as standards the protein purified in our laboratory. The immunoassay was validated by comparison with a fluorescence enzyme immunoassay (FEIA) using monoclonal antibodies (Hermans et al., 1998). Albumin and urea were quantified by the Beckman Synchron CX5 Delta Clinical System. The reproducibility of protein determination in NALF was assessed in four healthy subjects (2 men and 2 women, aged 26-8-59 years) who provided twice a day for five consecutive days a NALF sample from both nostrils. The mean NALF protein concentrations in these subjects ranged from 89 to 248 μg/l for CC16 and from 6.8 to 18.8 mg/l for albumin. The coefficients of variation (CVs) of CC16 and albumin determination in the 10 samples from each nostril averaged 42.6 % (range, 29.0-58.2 %) and 35.2 % (range, 16.7-55.1 %), respectively. Adjustment for the urea concentrations yielded rather similar values with a mean CV of 42.8% for CC16 and of 33.2% for albumin. These overall CVs appear quite acceptable considering that they integrate the analytical variation of immunoassays (CVs between 5-10%), the variable recovery of proteins from the nasal epithelial lining fluid (ELF) and probably also some biological variations in the ELF composition in the course of the experiment (5 days). Of note, the mean protein concentration ratio between the two nostrils, even after adjustment for urea, was not necessarily equal to 1 but ranged from 0.91 to 1.55 for CC16 and from 0.88 to 1.37 for albumin. This means that the NALF has to be sampled in the two nostrils for a reliable assessment of the nasal epithelium integrity of an individual.

Total and specific IgE analysis

Total and aeroallergen-specific IgE (house-dust mite, cat epithelium, dog dander, mould, tree pollen, grass pollen and herbaceous pollen mixture) concentrations in serum were determined using the Immulite® IgE kit (Diagnostic Products Company, Los Angeles, CA, USA). A sensitization against aeroallergen-specific IgE was defined as a serum concentration>0.35 kIU/l. For total serum IgE, we used the same cut-off of 30 kIU/l as in our previous studies. This cut-off value was selected because it approximated the 50^{th} percentile (27.5 kIU/l) or the geometric mean (27.3 kIU/l) of the adolescents in our study who had no detectable aeroallergen-specific IgE in serum. This low total serum IgE threshold was thus used to rule out most subjects likely to be sensitized to aeroallergens (Kerkhof et al, 2003). Concentrations of proteins in NALF were adjusted for the variable dilution of the epithelial lining fluid (ELF) using the NALF/plasma concentration ratio of urea (Cavalieri et al, 1986; Kaulbach et al, 1993).

Statistical analyses

Except for age and cumulative pool attendance (CPA), all continuous variables were log-transformed before statistical analysis.

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. Univariate associations were assessed using the Pearson's correlation coefficient. Multiple linear regression analysis was used to identify factors associated with biomarker concentrations in NALF.

Table 1. Characteristics of adolescents.

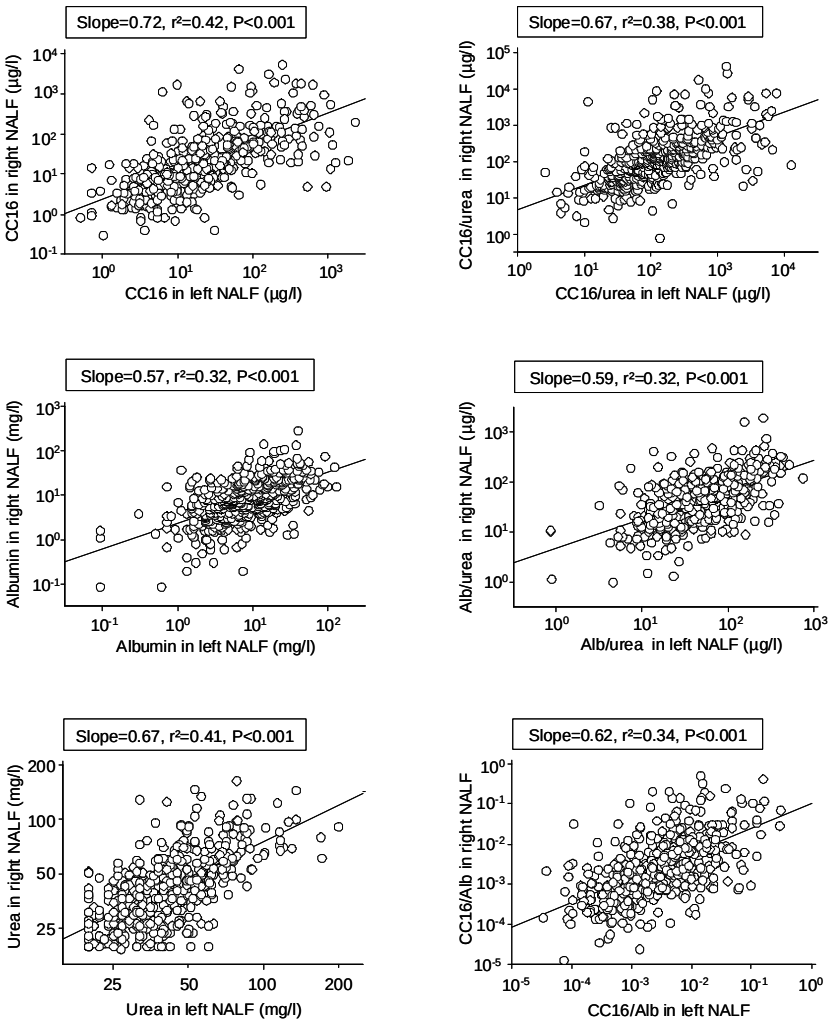
	Girls N=263	Boys N=211	p
Age, mean (SD), years	15.4 (0.78)	15.6 (0.93)	0.18
BMI, mean (SD), Kg/m2	20.7 (3.06)	20.5 (2.86)	0.50
Parental asthma, N° (%)	43 (16.4)	20 (9.5)	0.03
Parental allergy, N° (%)	105 (39.9)	69 (32.7)	0.11
Birth weight, mean (SD), g	3218 (545)	3432 (557)	<0.001
Breastfeeding, N° (%)	175 (66.5)	142 (67.3)	0.86
Day-care attendance, N° (%)	72 (27.4)	59 (28.0)	0.89
Smoking, active, N° (%)	20 (7.6)	18 (8.5)	0.71
Smoking during pregnancy, N° (%)	35 (13.3)	22 (10.4)	0.34
Parental smoking at home, N° (%)	96 (36.5)	73 (34.6)	0.67
Number of older siblings, mean (SD)	0.97 (0.94)	0.97 (1.03)	0.94
House cleaning with bleach, N° (%)	72 (27.4)	61 (28.9)	0.71
Mould on bedroom wall, N° (%)	20 (7.6)	13 (6.2)	0.54
Living at <100 m from a busy road, N° (%)	49 (18.6)	44 (20.9)	0.54
Exposure to pets since birth, N (%)	25 (9.5)	27 (12.8)	0.25
Indoor chlorinated pool			
Ever, N (%)	223 (56.0)	175 (82.9)	0.16
CPA, median (IQR), hours	302 (158-549)	271 (121-526)	0.21
Outdoor residential chlorinated pool			
Ever, N (%)	47 (17.9)	41(19.4)	0.69
Median (IQR), hours	192 (81-450)	192 (54-640)	0.78
Outdoor chlorinated pool during holidays			
Ever, N (%)	133 (50.6)	112 (53.1)	0.13
Median (IQR), hours	126 (45-280)	186 (65-385)	0.22
Total serum IgE, median (IQR), kIU/l	32.0 (15.2-103)	60.1 (22.0-44.8)	<0.001
Sensitization to aeroallergens			
At least one aeroallergen, N° (%)	77 (29.3)	84 (39.8)	0.016
House dust mite, N° (%)	55 (20.9)	68 (32.2)	0.005
High sensitization, N° (%)	22 (8.4)	31(14.7)	0.03
Low sensitization, N° (%)	33 (12.5)	37 (17.5)	0.13
Cat, N° (%)	23 (8.8)	25 (11.8)	0.27
Pollen, N° (%)	33 (12.5)	40 (19.0)	0.055
Ever diagnosed respiratory diseases			
Asthma, N° (%)	15 (5.7)	17 (8.1)	0.31
Hay fever, N° (%)	25 (9.5)	17 (8.1)	0.58
Allergic rhinitis, N° (%)	28 (10.6)	19 (9.0)	0.55
Bronchitis, N° (%)	110 (41.8)	81 (38.6)	0.47
Bronchiolitis, N° (%)	39 (14.8)	28 (13.3)	0.64
Sinusitis, N° (%)	48 (18.3)	38 (18.1)	0.97
Cold, N° (%)	75 (28.5)	50 (23.8)	0.25

Regression models were run by testing a total of 23 independent variables, including among others, age, body mass index (BMI), breastfeeding, tobacco smoking, exposure to environmental tobacco smoke (ETS), vicinity to a busy road, aeroallergen sensitization and doctor-diagnosed diseases of the upper airways. In these analyses, attendance of the different types of chlorinated pool (indoor, outdoor residential, outdoor during holidays) was stratified in three categories of increasing lifetime CPA (CPA<100, CPA 100-500 and CPA>500 hours). These categories were transformed in dummy variables using as referents subjects who never swam in the considered swimming pool. We used backward logistic regression models to calculate the odds ratios (ORs) of being sensitized against house dust mite (HDM) or pollen according to the levels of CC16, albumin and of the CC16/albumin ratio in NALF. P values were two-sided and results were considered as statistically significant at P values below 0.05.

RESULTS

The characteristics of participants are presented in Table 1. With the exception of parental asthma, which was more frequent in girls than in boys, there were no significant sex differences in the major risk factors of allergic or respiratory diseases. Prevalences of doctor-diagnosed asthma, hay fever and allergic rhinitis also did not differ between sexes. The only noticeable differences concerned the total serum IgE levels and the sensitization rates to aeroallergens and especially to HDM, which were both significantly higher in boys than in girls. In a first step, we analyzed our data by comparing the concentrations biomarkers in the NALF from the two nostrils. In order to assess the influence of the variable dilution of the ELF during nasal lavage, comparisons were made with concentrations of CC16 and albumin, unadjusted or adjusted for the NALF/plasma urea ratio.

Figure 1. Correlations between biomarkers concentrations in right and left nostrils.



As shown in Figure 1, concentrations of CC16 and albumin expressed per liter as well as their ratio in the two nostrils were rather well correlated (CC16, r=0.65; albumin, r=0.57; CC16/albumin ratio, r=0.58, all p<0.001)

with however a considerable inter-individual variations over more than four orders of magnitude. Concentrations of urea in the NALF from both nostrils were also well correlated ($r=0.64$, $p<0.001$). Adjustment of protein concentrations for the NALF/plasma ratio did not improve these correlations (CC16, $r=0.36$; albumin, $r=0.57$, $p<0.001$), nor did this adjustment reduce the inter-individual variations. We therefore pursued our analyses by using the means of values measured in the NALF from the two nostrils, with and without adjustment for the NALF/plasma urea concentration ratio.

Table 2 compares the levels of biomarkers in NALF between boys and girls. The concentration of urea in NALF was slightly higher in boys than in girls but there was no significant sex difference in the NALF/plasma urea ratio. Concentrations of CC16 in NALF, adjusted or not for the NALF dilution, did not differ significantly between boys and girls. This is in contrast to the albumin concentration, which on average was about 30% greater in boys than in girls.

As shown in Table 3, the determinants of epithelial markers in NALF differed noticeably between the two sexes. In boys, the concentration of CC16 in the NALF, adjusted or not for the NALF/plasma urea ratio, correlated negatively with age and positively with parental smoking. Also in boys, the concentration of albumin in NALF, whether adjusted or not for the urea ratio, decreased with the number of siblings, the BMI and the proximity to a busy road. Albumin in the NALF of boys also decreased with age but this association disappeared after adjustment for the urea ratio. The only positive association with NALF albumin, suggesting an increased epithelial permeability, was that between the urea-adjusted concentration of albumin and the lifetime attendance of public chlorinated pools (CPA>500 hours). Quite interestingly, the CC16/albumin ratio in the NALF of boys, an index integrating changes in CC16 secretion and nasal epithelium permeability, decreased dose-dependently with the lifetime attendance of indoor

chlorinated pools (CPA 100-500 and CPA>500 hours). Like the CC16 concentration, the CC16/albumin ratio in the NALF of boys, showed a

Table 2. Biomarkers in the NALF of girls and boys.

Parameters	Boys (N=211)	Girls (N=263)	P
Urea (mg/l)			
crude	43.5 (33.0-59.9)	40.0 (31.1-51.0)	0.03
mg/mg plasma urea	0.18 (0.13-0.23)	0.17 (0.13-0.23)	0.94
CC16 (µg/l)			
crude	24.5 (8.80-67.7)	18.7 (7.50-70.4)	0.33
adjusted the NALF/plasma urea ratio	149 (59.9-327)	121.9 (46.0-370)	0.30
Albumin (mg/l)			
crude	10.5 (6.4-19.8)	6.90 (3.61-14.4)	<0.001
adjusted the NALF/plasma urea ratio	57.2 (35.8-107)	41.0 (22.8-81.5)	<0.001
CC16/albumin ratio ($\times 10^4$)	25.3 (9.01-77.7)	32.9 (10.6-98.7)	0.08

Concentrations are expressed as median with interquartile range. CC16: Clara Cell Protein.

positive association with parental smoking. In girls, by contrast, the CC16 concentration in NALF, irrespectively of the adjustment for the urea ratio, decreased with both parental smoking and the vicinity of a busy road. There was also a negative association between the urea-adjusted CC16 concentration in the NALF of girls and the lifetime attendance of residential outdoor chlorinated pools (CPA>500 hours). None of the other tested factors was associated with albumin or the CC16/albumin ratio in the NALF of girls.

When tested separately, the concentrations of CC16 and albumin in NALF, expressed per liter or adjusted for the NALF/plasma urea ratio, showed no significant associations with the risk of sensitization to HDM or pollen. However, as shown in Table 4, the CC16/albumin ratio in the NALF

of boys, whether tested as a continuous variable or stratified in tertiles, was inversely related to the risk of sensitization to HDM, especially when total serum IgE was >30 kIU/l. Among boys with higher total serum IgE, those in the lowest tertile of the CC16/albumin ratio in NALF were three times more likely to be sensitized against HDM than those in the highest tertile. There was also a tendency for boys in the lowest tertile of the NALF CC16/albumin ratio to be more frequently sensitized against pollen, an association again emerging when total serum IgE >30 kIU/l. Such associations were not seen in girls, even among those with higher serum IgE.

DISCUSSION

Our study shows that concentrations of CC16 and albumin in the NALF greatly vary despite adjustment for the variable dilution of the recovered ELF on the basis of the NALF/plasma urea ratio. Variations were just important as for the CC16/albumin ratio, which theoretically should be independent of the dilution of ELF. There were also some variations between the two nostrils, the protein concentrations in the right nostril being on average about 30% lower than in the left one. We think that this systematic difference stems from the fact that the left nostril was in most cases the second one sampled with presumably a better ELF recovery because of some training effect or of a carry-over of proteins from the first nostril. The adjustment made for the NALF/plasma urea ratio ensured a complete elimination of the variations due to the variable recovery of ELF proteins. Variations in the levels of albumin and CC16 in NALF observed under these conditions can thus be assumed to reflect true variations in the permeability (albumin) or the cellular integrity

Table 3. Factors associated with the concentrations of biomarkers in NALF of boys and girls.

	Boys			Girls		
Dependent variable	Independent variable	Regression coefficient (95% CI)	p	Independent variable	Regression coefficient (95% CI)	p
Urea (mg/l)	Allergic rhinitis	-0.10 (-0.18 to -0.02)	0.02			
	Birth weight	-0.41 (-0.72 to -0.09)	0.01			
NALF/plasma urea ratio	Number of older siblings	-0.02 (-0.05 to -0.002)	0.048	Residential outdoor pool (CPA>500 h)	0.13 (0.02 to 0.24)	0.02
CC16 (µg/l)	Age	-0.16 (-0.25 to -0.06)	0.002	Parental smoking	-0.22 (-0.40 to -0.04)	0.01
	Parental smoking	0.19 (0.004 to 0.38)	0.049	Living<100 m from a busy road	-0.22 (-0.44 to -0.005)	0.045
CC16 adjusted *	Parental smoking	0.18 (0.01 to 0.35)	0.042	Living<100 m from a busy road	-0.21 (-0.41 to -0.01)	0.04
	Age	-0.16 (-0.25 to -0.07)	0.001	Parental smoking	-0.25 (-0.40 to -0.07)	0.004
				Residential outdoor pool (CPA>500 h)	-0.43 (-0.83 to -0.02)	0.04
Albumin (mg/l)	Number of older siblings	-0.07 (-0.12 to -0.03)	0.004			
	BMI	-0.91 (-1.80 to -0.02)	0.046			
	Living <100 m from a busy road	-0.15 (-0.27 to -0.02)	0.03			
	Age	-0.06 (-0.12 to -0.01)	0.03			
	Mold on bedroom walls	-0.23 (-0.44 to -0.02)	0.03			
Albumin adjusted*	Number of older siblings	-0.07 (-0.11 to -0.02)	0.008			
	BMI	-0.15 (-0.28 to -0.03)	0.02			
	Living <100 m from a busy road	-0.13 (-2.35 to -0.65)	0.001			
	Public indoor pool (CPA>500 h)	0.13 (0.01 to 0.25)	0.04			
CC16/albumin ratio	Public indoor pool (CPA>500 h)	-0.42 (-0.68 to -0.17)	0.001			
	Public indoor pool (CPA 100-500h)	-0.24 (-0.45 to -0.03)	0.02			
	Parental smoking	0.26 (0.06 to 0.45)	0.01			

*All biological parameters were normalized by logarithmic transformation. *[a]Adjusted for NALF/plasma urea ratio*

Table 4. Risks of sensitization to house dust mite or pollen with decreasing CC16/albumin ratio in the NALF of girls and boys with low or high total serum IgE.

		All	Boys			Girls		
Sensitization	CC16/ albumin ratio	n= 474	All n=211	<30kIU/l n=63	>30kIU/l n=148	All n=263	<30kIU/l n=129	>30kIU/l n=134
		OR (95% CI)[a]	OR (95% CI)[e]	OR (95% CI)	OR (95% CI)[e]	OR (95% CI)[b]	OR (95% CI)[c]	OR (95% CI)[d]
Dermatophagoïdes	Continuous	1.34 (0.97-1.87)	1.86 (1.14-3.03)	-	1.84 (1.12-3.01)	1.10 (0.69-1.76)	0.88 (0.29-2.63)	1.16 (0.67-2.02)
	Tertile 1	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	Tertile 2	1.17 (0.66-2.06)	1.82 (0.77-4.29)	-	1.64 (0.65-4.16)	1.34 (0.62-2.91)	1.26 (0.21-7.47	1.15 (0.47-2.83)
	Tertile 3	1.67 (0.96-2.88)	2.54 (1.11-5.84)	-	2.83 (1.14-7.01)	1.12 (0.50-2.52)	0.76 (0.13-4.46)	1.48 (0.54-4.08)
P for trend		0.03	0.02	0.58	0.02	0.51	1.00	0.17
		OR (95% CI)[f]	OR (95% CI)[i]	OR (95% CI)	OR (95% CI)[j]	OR (95% CI)[g]	OR (95% CI)[h]	OR (95% CI) [i]
Pollen	Continuous	1.29 (0.87-1.92)	1.46 (0.85-2.54)	1.01 (0.12-8.42)	1.54 (0.85-2.78)	1.20 (0.67-2.15)	0.75 (0.16-3.64)	1.40 (0.73-2.69)
	Tertile 1	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
	Tertile 2	1.48 (0.75-2.89)	1.16 (0.45-3.01)	-	0.81 (0.27-2.45)	1.49 (0.59-3.78)	0.67 (0.05-9.61)	1.55 (0.56-4.28)
	Tertile 3	1.37 (0.69-2.70)	2.00 (0.84-4.77)	-	2.00 (0.75-5.34)	0.97 (0.35-2.67)	0.50 (0.03-7.15)	1.28 (0.40-4.06)
P for trend		0.21	0.095	0.89	0.07	0.79	0.54	0.32

The continuous models are based on log transformed data.
[a]Adjusted for BMI, pets, parental asthma and gender;[b]Adjusted for parental asthma, breastfeeding, pets and day-care attendance; [c]Adjusted for house cleaning with bleach and mould on bedroom walls; [d]Adjusted for pets and breastfeeding; [e]Adjusted for BMI, pets and living<100 m from a busy road; [f]Adjusted for pets and gender; [g]Adjusted for breastfeeding, pets, day-care attendance and living<100 m from a busy road; [h]Adjusted for parental asthma, breastfeeding and day-care attendance; [i]Adjusted for pets; [j]Adjusted for pets and day care attendance.

(CC16) of the nasal epithelium.

One of the most prominent findings in our study is the inverse association found between the CC16/albumin ratio in the NALF and the risk of sensitization to aeroallergens, in particular to house dust mite. The fact that this association emerges only in boys is interesting from a mechanistic point of view. This suggests indeed that the association between the NALF CC16/albumin ratio and the risk of allergic sensitization is driven by interactions with gender-related risk factors. Clearly, one interacting factor is the atopic status assessed on the basis of the total serum IgE level that was significantly higher in boys than in girls (by 25% on average). Total serum IgE strongly influences the association between the CC16/albumin ratio in NALF and the risks of allergic sensitization to aeroallergens, which were increased only among boys with serum IgE>30 kUI/l. Another source of interaction appears to be related to the response of nasal epithelium to some environmental stressors. The CC16/albumin ratio in NALF of boys was indeed significantly lower than that of girls, a difference that might be linked to the exposure to chlorinated pools. While no predictor could be identified for the NALF CC16/albumin ratio in girls, the attendance of public chlorinated pools emerged as a very significant predictor decreasing dose-dependently the CC16/albumin ratio in the NALF of boys. These findings suggest thus that changes of the nasal epithelium barrier facilitating atopic sensitization might consist in an increased permeability associated with functional deficit of the nasal epithelium, with in particular a decrease secretion of the anti-inflammatory CC16 protein. In other terms, this suggests that only stressors or combinations of stressors capable of causing such epithelial defects are implicated in the development of allergic sensitization. Our study points to chlorination products as important drivers of this type of epithelial defect but of course other stressors such as dry air, ambient air pollutants or infectious diseases might also contribute to these epithelial changes (Simoni et al 2010).

Predictors of CC16 and albumin levels in NALF were mostly factors that have been associated with the risk of respiratory diseases, including age, gender, ETS, BMI, traffic-related air pollution and attendance of chlorinated swimming pools (Bernard et al, 2008; 2009). Associations found between these predictors and the NALF biomarkers were however not necessarily pointing to the same direction as those reported with respiratory diseases and in some cases they were inconsistent between sexes. While in girls parental smoking was associated with a decrease of NALF CC16, suggesting some epithelial damage, the opposite associations were seen in boys exposed to ETS who had higher CC16 and CC16/albumin ratio in NALF. The same inconsistency was observed with the vicinity of a busy road. In girls, this factor was associated with a decrease of NALF CC16, suggesting an impaired CC16 secretion, while boys it was associated with a decrease of NALF albumin, which points to a more efficient barrier function. These inconsistencies might reflect gender-related differences in the response of the nasal epithelium to ETS or the ambient air pollution. Women for instance are known to be more vulnerable to the harmful effects of tobacco smoke than men when being actively or passively exposed to tobacco smoke (Dransfield et al, 2006; Li et al, 2000; Chen et al, 2005). These opposite associations might also merely reflect the influence of some unidentified confounders or a distortion of our analyses by the lack of quantitative exposure data. The apparently protective effects of BMI and number of siblings on the permeability of nasal epithelium to albumin observed in boys might also arise from confounding.

Actually, the predictor that was the most consistently associated with nasal epithelium alterations in both sexes was the attendance of chlorinated swimming pools. In both sexes, changes observed with CC16 (decrease) or albumin (increase) were all suggestive of functional or structural alterations of the nasal epithelium. Attendance at chlorinated pools was also the only predictor to display a significant dose-response relationship, in particular

with the CC16/albumin ratio. There were however still some inconsistencies between sexes as NALF biomarkers were associated in boys with indoor public pools and in girls with outdoor chlorinated pools. We think that these inconsistencies might reflect differences in the exposure patterns or in the deposition of chlorination products in the nasal cavity. Because of their higher activity, boys might behave differently from girls when attending indoor or outdoor pools. For instance, it is possible that boys remain less time in the water of residential outdoor pools than girls and consequently are less exposed to the chlorine-based irritants building up at the surface of the pool. By contrast, in indoor public pools, where it is really possible to develop own swimming capacity, boys might train more intensively than girls and thus be more exposed chlorination products. These sex differences in the response of the nasal epithelium to chlorinated pools might also be linked to differences in the anatomic features of the nasal cavity. Women are indeed known to have smaller nasal dimensions than men, which may favor the deposition of aerosolized and gaseous chlorination products in the nasal cavity. A gender difference also appears to exist for the irritating effects of chlorine in swimming pools. A recent study among teenagers training in swimming pools shows that the attendance of outdoor pools increases the risk of asthma in girls but not in boys (Siegel et al, 2010), which is consistent with our study showing a decrease of NALF CC16 only in girls exposed to outdoor pool. In line with these observations, we found significant gender differences in the determinants of CC16 and albumin in NALF.

In agreement with previous studies based on serum CC16 and surfactant-associated proteins (Carbonnelle et al, 2002; Bernard et al, 2003; Lagerkvist et al, 2004; Bernard et al, 2007), our data provide further evidence that chlorination products can affect the cellular integrity and permeability of airways epithelium. A decrease of CC16 as found in girls having attended outdoor pools can be interpreted only as the consequence of a loss of CC16-secreting cells in the nasal cavity since the urea corrected

concentrations of CC16 in NALF are approximately 10 times higher than the serum concentrations of CC16. The higher concentrations of albumin in the NALF of boys who regularly visited indoor chlorinated pools testify to an increased epithelial permeability, which could result from the tight junction disruption caused by hypochlorous acid and chloramines. While no association was found with the concentrations of CC16 and albumin in NALF when tested separately, in the NALF of boys, the ratio between the proteins correlated with an increased risk of sensitization to aeroallergens. This finding suggests that changes of the nasal epithelium facilitating atopic sensitization might consist in an increased epithelial permeability associated to a decreased secretion of proteins with anti-inflammatory properties such as CC16. In other terms, this suggests that only stressors or combinations of stressors capable of causing such epithelial defects are implicated in the development of allergic sensitization. Our study points to chlorination products as important drivers of this type of epithelial defect but of course other stressors such as dry air, ambient air pollutants or infectious diseases might also contribute to these epithelial changes (Simoni et al, 2010; Walinder et al, 2000).

Our study has some limitations. The first limitation is the relatively small size of the subpopulations obtained after stratification for sex, which has reduced the statistical power of some analyses. The initial cohort included more than 800 subjects but we lost about half of them after excluding subjects with signs of rhinitis or who were under medication for allergic diseases at the time of examination. The second limitation is the lack of exposure levels data, in particular for environmental risk factors. It was of course impossible to perform air measurements for assessing the individual exposure to ETS or to chlorination products and *a fortiori* to obtain such data for the past exposure when adolescents were infants or children.

In conclusion, our study shows that the concentrations CC16 and albumin in NALF might serve as noninvasive biomarkers to detect

nasal epithelium damage caused by air pollutants and other stressors. In our adolescents, defects in nasal epithelium detected by the CC16/albumin ratio appear to be mostly caused by the attendance of chlorinated pools. In boys, these effects were associated with an increased risk of sensitization to house dust mite.

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3.2 Study 2: Nasal epithelium biomarkers in young children: Associations with allergic sensitization and environmental stressors.

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

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

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ABSTRACT

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 μg/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.

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 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 ozone[9] 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 non-invasive 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 non-invasive 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 thirty 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 both nostrils as described previously[21].

CC16 was quantified by a semi-automated non-isotopic 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) 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 to 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 hours 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).

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.

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

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.

Table 2. Biomarkers in the NALF of boys and girls.

Parameters	Girls (N=146)	Boys (N=155)	P
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.

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 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 CC16 (2.59, 95 % CI, 1.15-5.82, p=0.02) 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

Table 3. Factors influencing the concentrations of urea, CC16 and albumin NALF of girls and boys.

		Girls (n=146)			Boys (n=155)	
Dependent Variable	Independent variable	Regression coefficient (95% CI)	P	Independent variable	Regression coefficient (95% CI)	p
						0.02
Log CC16 (µg/l)	Frequent colds	-0.24 (-0.40 to -0.07)	0.006	Age	0.27 (0.03 to 0.50)	0.03
	Parental smoking	0.24 (0.07 to 0.41)	0.007	Bleach	-0.21 (-0.39 to -0.03)	
	CPA>100 h	-0.18 (-0.35 to -0.02)	0.03			
Log CC16 /urea ratio	CPA>100 h	-0.22 (-0.40 to -0.05)	0.01	Age	0.34 (0.09 to 0.58)	0.008
	Probiotics	0.04 (0.01 to 0.08)	0.02	Frequent colds	-0.17 (-0.33 to -0.01)	0.043
	Parental smoking	0.19 (0.01 to 0.38)	0.04	Bleach	-0.26 (-0.45 to -0.07)	0.008
	Living in Liège	0.19 (-0.01 to 0.38)	0.04			
Log Albumin (mg/l)	Probiotics	-0.04 (-0.07 to -0.01)	0.007	Frequent cold	0.13 (0.004 to 0.27)	0.044
	HDM IgE	0.24 (0.0004 to 0.47)	0.049	Living in Liège	0.17 (0.04 to 0.30)	0.01
	Frequent colds	-0.17 (-0.31 to -0.03)	0.02	Pollen IgE	0.23 (0.05 to 0.40)	0.01
Log Albumin/urea ratio	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 to 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 h	-0.22 (-0.49 to -0.01)	0.04	Age	0.31 (0.01 to 0.61)	0.044
	Age	0.38 (0.08 to 0.69)	0.02			

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

Table 4 Risks of sensitization to house dust mite according to increasing levels of albumin and decreasing levels of CC16 and of the C16/albumin ratio in the NALF of all children, or of girls and boys separately.

	All (OR 95% CI)	P	Girls (OR 95% CI)	P	Boys (OR 95% CI)	P
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
P 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)	0.05	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)	1	1.91 (0.45-8.14)	0.38	1.27 (0.38-4.27)	0.70
P for trend	0.19	0.10	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
P 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
P 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
P for trend	0.08		0.09		0.97	

CC16, Clara cell protein

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 non-invasively 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 previously[21] 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 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 adolescents[21] (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 vs. 21.3 µg/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 x 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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4. Discussion and perspectives

The purpose of our study was to analyze the correlations between exposure to environmental stressors, nasal epithelium lesions and allergic sensitization.

In order to assess the damage to the nasal mucosa we decided to use some specific biomarkers in nasal lavage, as this technique of sampling is not invasive. The biomarkers chosen are the CC16 and the albumin. The CC16 of the nose is produced by pseudostratified epithelium cells, as in the nasal mucosa Clara cells are absent. A reduction of concentration of CC16 in the nose can be caused by the destruction of the epithelial cells that produce the protein or by the leakage of the protein across the disrupted epithelial barriers of the nasal cavity or by a decreased production/secretion. Albumin being not produced by the nasal epithelium, it can only originate from the nasal blood as a consequence of transudation or exudation from capillaries.

The results, that are common to both studies, show that nasal epithelium defects as reflected by a decrease of the CC16/alb ratio in NALF are associated with an increased risk of sensitization to the house dust mite (HDM). Unfortunately, since both studies are cross-sectional, we cannot determine the direction of causality in these associations. The question that remains to be resolved is whether the damage to the nasal epithelium is due to the action of proteolytic allergens or if it is a pre-existing damage facilitating the penetration of allergens across the epithelial barrier.

Several studies have suggested that the cause of allergic sensitization may be an alteration of the epithelial barrier. While proteolytic activity has been shown for some allergens (Jeong KY et al, 2012), the exposure to certain toxic or irritating induces changes in the values of the biomarkers that reflect the epithelial permeability (Calderon-Garciduenas L et al, 2001).

For this reason, we considered several factors that can alter the balance of the epithelial barrier and we analyzed their action on the nasal biomarkers. In both studies, we were able to highlight the occurrence of certain environmental factors that seem to influence the epithelial permeability: the distance from a busy road, the nasal pathologies, the parental smoking and the chlorinated pool attendance. While the first three cases were, in some way, predictable, given the proven harmful action of air pollution, passive smoking and inflammation on the respiratory epithelium (Madsen C et al, 2008; Sokolo Gedikondele J et al, 2011), the epithelial damage related to the swimming pool attendance seems to be a new and interesting outcome. This result can be explained by the action of the byproducts of chlorination on the cells of the respiratory epithelium. Chloramines are indeed powerful oxidants that react with sulphydryl groups of proteins, causing cell retraction, disruption of tight junction and an increase in endothelial and epithelial permeability (Carbonelle S et al, 2002). Recent studies have shown that elite swimmers have a higher incidence of allergic and non-allergic rhinitis and, more generally, of symptoms of inflammation of the upper and lower airways that other athletes or control subjects. Some symptoms might be related to the intensity of the sport, but intense exercise can not explain the persistence of symptoms and epithelial damage after finishing the exercise in the pool (Bougault V et al, 2009). Moreover the evidence of epithelial damage is observed only in the case of chlorinated pools attendance, not in the case of swimming in cupper-silver.

Another common aspect to the two studies is the presence of different results, sometimes even contradictory, between the two sexes. It would be possible to evoke the anatomical differences between the sexes, with females having a smaller size of the nose (even though, when adjusted for the difference of body size, the difference between males and females seem to disappear (Pohunek P,2004)), and the physiological differences, which are the result of the action of sex hormones on the nasal epithelium (this

consideration cannot of course be applied to the population of children), however, these considerations do not seem sufficient to explain some inconsistencies, such as the exposure to environmental tobacco smoke and the proximity to a busy road, which show a negative effect on males and beneficial on females. These results are perhaps to be considered due to the action of some cofactors, that we have not been able to identify, or to the small number of subjects in each subgroup and the lack of information concerning the degree of exposure to environmental toxics.

Comparing the results of the two studies, we also noticed a big difference between the two populations: the concentrations of biomarkers. The children in fact have lower amounts of CC16 and higher ones of albumin. The first difference could be interpreted as being due to the reduced dimensions of the nose of children and, consequently, of the mucosa, which thus has a lower number of cells secreting the CC16. If so, we would have found even lower concentrations of albumin. On the contrary this is 4 times higher in children and this data can be interpreted, in our opinion, as the result of a higher epithelial permeability during the childhood. In fact during childhood the nasal epithelium in children is exposed to conditions that are no longer detectable in adulthood. First, among adolescents and children, there are large differences in anatomy. From the age of two years, pneumatization of the paranasal sinuses begins and will last until puberty (Scuderi AJ et al, 1993; Shah RK et al, 2003). One of the most important roles of the sinuses is the production of nitric oxide (NO) and it was shown that most of NO present in the nasal cavities comes from the paranasal sinuses. NO contribute significantly to non-specific host defence against bacterial, viral and fungal infections. It inhibits some metalloproteins oxidase and catalase, the lipid peroxidation and thus the formation of proinflammatory lipids and affects positively mucociliary clearance. It is therefore considered that NO is responsible for maintaining a sterile environment inside the sinuses. Being also a potent vasodilator, it seems to

play a role in the regulation of nasal airway resistance to airflow and in the process of heating and humidification of the inspired air (Maniscalco M et al, 2007). Since the paranasal sinuses in children are less well developed, probably the nasal mucosa can not fully benefit from the protective action of NO. It should be also added that infections among children by rhinovirus (RV) are very frequent. *In vitro* studies have demonstrated that RV infection of cells of the nasal epithelium causes a decrease in the expression of some structural proteins of tight junctions and adherens junctions, such as the claudin-1, the occluidin, the ZO-1 and the E-cadherin, and consequently an increase in epithelial permeability (Yeo NK and Jang YJ, 2010). These results, together with a possible immaturity of the nasal epithelium, may help explain the different concentration of biomarkers in NALF among children and adolescents and the epithelial vulnerability to the allergic sensitization at a very young age.

To the best of our knowledge, our study is the first to have investigated the nasal epithelium integrity in populations of school children or adolescents. We were also the first to compare the urea-adjusted concentrations of CC16 and albumin between the two nostrils. Our studies present however several limitations. First, because of practical reasons linked to the very young age of children, we could not perform an extensive exploration of the nasal cavity. The determination of the nasal patency would have certainly provided useful information to understand the differences between children and adolescents. But even the measurement of the peak nasal inspiratory flow revealed very difficult in our 5-years old children who often did not understand the difference between exhaling and inhaling though the nose only. Another limitation is the relatively small numbers of subjects that were achieved when stratifying the population for gender and/ or total serum IgE. This reduction in the number of subjects has resulted in a diminished statistical power of our analyses and some associations, in particular concerning pollen sensitization, might have

reached the level of statistical significance if we had had access to larger populations. Because of this limitation, we could not assess the associations with less frequent allergies such as allergies to cat or dog. Another limitation is the fact that our studies were cross-sectional, which, as mentioned above, prevents us to assess the causality of associations emerging between the various events. We cannot determine whether the observed epithelial changes are the cause or the consequence of allergic sensitization. There are two possible explanations for the fact that in our studies nasal epithelial defects were significantly associated with HDM sensitization but not with pollen sensitization. The first is that the HDM causes more damage to nasal epithelium because this allergen would have a stronger proteolytic capacity as compared to the pollen allergens. The second is that to promote allergic sensitization, defects in the nasal epithelium require a concomitant and sufficient exposure to the allergen, a condition more easily met with a perennial allergens such as HDM than with seasonal allergens linked to pollination. A last limitation of our studies is that exposure assessment was entirely based on questionnaires filled by the parents, with the risk of memory bias.

If biomarkers in NALF can provide a non-invasive assessment of the nasal epithelium function or integrity, the exact nature of the defects evidenced by these biomarkers remains to be elucidated. The best approach would be of course the "direct" observation of the epithelial changes induced under experimental conditions. Various studies on cell cultures, obtained from nasal biopsies, were performed to analyze the action of environmental toxics. Carson JL et al. (2010) observed an accelerated ciliary beat frequency in cultured human nasal epithelium obtained from smokers and individuals exposed to secondhand tobacco smoke. These authors also reported a phenotypic modification favoring the expression of non-ciliated epithelial cell types in specimens deriving from smokers and secondhand smoke-exposed subjects. Auger F et al. (2006) exposed cultures of nasal cells for

24h to diesel exhaust particles and Paris urban air particles and observed an increase in local inflammation, expressed as an increase in certain inflammatory mediators, such as IL-8, IL-6 and GM-CSF. With regard to exposure to products of chlorination, no study has been carried out so far *in vitro* cultures of nasal cells. An experimental study was however performed on lung cells exposed to trichloramine (Schmalz C et al, 2011). The results show a harmful effect of trichloramine at concentrations higher than those normally present in the air of indoor swimming pools. The authors concluded that the harmful effect on the epithelium is not due solely to trichloramine, but to a mixture of trichloramine and other disinfection by-products present not only in the air, but also in aerosol on the surface of the water. It would therefore be interesting to expose a culture of nasal cells to these substances and to assess the level of the epithelial damage. More complex *in vitro* models might also be used to evaluate permeability changes of the nasal epithelium by measuring the flux of Evans blue (EB) dye-labeled bovine serum albumin through an endothelial/epithelial cell nasal bilayers (Wang L et al, 2013).

Experimental studies might also be conducted to measure the trans-epithelial passage of allergens, as Rusznak C et al. did in 1999. Exposing primary cultures of human bronchial epithelial cells to cigarette smoke in the absence or presence of Derp extracts, these authors observed an increased passage of Derp through the epithelium in the cultures exposed to tobacco smoke as compared to non-exposed cultures. A study of this kind could help to understand if the epithelial damage induced, for instance by chlorination products, might promote the penetration of allergens.

Another avenue of research would be the immunohistochemical exploration of biopsies of nasal epithelium from subjects exposed to various environmental stressors (e. g. with the immunostaining of CC16 and proteins of the tight junctions such as claudin-1, the occludin and the ZO-1)

In conclusion, our studies suggest that the analysis of the concentrations of CC16 and albumin in NALF might be useful to assess non-invasively the integrity of the nasal epithelium. Further studies are needed however to identify the type of epithelial defects reflected by these NALF biomarkers and their exact biological significance in particular with respect to the risks of allergic sensitization.

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Impact of Chlorinated Swimming Pool Attendance on the Respiratory Health of Adolescents

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Impact of Chlorinated Swimming Pool Attendance on the Respiratory Health of Adolescents

WHAT'S KNOWN ON THIS SUBJECT: Outdoor or indoor swimming pools can increase asthma risks, but their overall influence on allergic diseases has not been evaluated.

WHAT THIS STUDY ADDS: This study shows that attendance at chlorinated swimming pools exerts a strong adjuvant effect that contributes significantly to the burden of asthma, hay fever, and allergic rhinitis during adolescence.

abstract

OBJECTIVE: The goal was to estimate the burden of allergic diseases associated with chlorinated pool exposure among adolescents.

METHODS: We examined 847 students, 13 to 18 years of age, who had attended outdoor or indoor chlorinated pools at various rates. Of them, 114 had attended mainly a copper-silver pool and served as a reference group. We measured total and aeroallergen-specific immunoglobulin E (IgE) levels in serum and screened for exercise-induced bronchoconstriction. Outcomes were respiratory symptoms, hay fever, allergic rhinitis, and asthma that had been diagnosed at any time (ever asthma) or was being treated with medication and/or was associated with exercise-induced bronchoconstriction (current asthma).

RESULTS: Among adolescents with atopy with serum IgE levels of >30 kIU/L or aeroallergen-specific IgE, the odds ratios (ORs) for asthma symptoms and for ever or current asthma increased with the lifetime number of hours spent in chlorinated pools, reaching values of 7.1 to 14.9 when chlorinated pool attendance exceeded 1000 hours. Adolescents with atopy with chlorinated pool attendance of >100 hours had greater risk of hay fever (OR: 3.3-6.6), and those with attendance of >1000 hours had greater risk of allergic rhinitis (OR: 2.2-3.5). Such associations were not found among adolescents without atopy or with copper-silver pool attendance. The population attributable risks for chlorinated pool-related ever-diagnosed asthma, hay fever, and allergic rhinitis were 63.4%, 62.1%, and 35.0%, respectively.

CONCLUSION: Chlorinated pool exposure exerts an adjuvant effect on atopy that seems to contribute significantly to the burden of asthma and respiratory allergies among adolescents. *Pediatrics* 2009;124: 1110–1118

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KEY WORDS

chlorine, swimming pool, childhood asthma, atopy, aeroallergens, hay fever, rhinitis

ABBREVIATIONS

CPA—chlorinated pool attendance
OR—odds ratio
PAR—population attributable risk
FEV_1—forced expiratory volume in 1 second
EIB—exercise-induced bronchoconstriction
IgE—immunoglobulin E

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Swimming pools commonly are disinfected through water chlorination with hypochlorite, chlorine gas, or chloroisocyanurates. These chlorine-based disinfectants, loosely referred to as chlorine, release in water hypochlorous acid, a nonselective biocide that oxidizes all forms of organic matter in pool water, which has advantages but also some disadvantages. Because of its strong oxidizing potential, chlorine can inactivate a wide spectrum of waterborne pathogens, but it also reacts with organs of swimmers in contact with pool water or air, causing irritation of the skin, eyes, and upper respiratory tract. In addition, when oxidizing organic matter from swimmers or other sources, chlorine releases a mixture of harmful byproducts, including irritants such as chloramines and haloacetic acids.[1–4]

Regulatory bodies and health authorities long have regarded these irritating effects of pool chlorine as a mere source of discomfort for swimmers, arguing that the dangers of chlorine, if any, must be weighed against the risks of inadequate disinfection and the health benefits of swimming.[1,5] This way of reasoning, which has dominated swimming pool management for several decades, is being increasingly challenged by reports of health problems among swimmers.[4] For several years, it has been known that elite swimmers have a higher prevalence of respiratory symptoms, asthma, and airway inflammation than do other athletes.[4] It was assumed that this poorer respiratory health of swimmers was the consequence of a selection bias attributable to the lower asthmagenicity of indoor swimming, compared with other sports. Researchers increasingly acknowledge that these respiratory problems may be attributable, at least in part, to chlorine used to disinfect pool water.[6–8]

Public concern about the dangers of pool chlorine, however, was particularly aroused when it was found that indoor chlorinated pools may be detrimental to the airways of children, causing epithelial damage and increasing asthma risk.[9–12] Studies by other investigators confirmed these respiratory effects of pool chlorine[13–15] and provided additional evidence that chlorinated pools may contribute to the development of allergic diseases.[16]

Initially, the respiratory problems of swimmers were attributed to the irritating effects of trichloramine, the gas that gives indoor pools their typical smell and can cause asthma in lifeguards.[17,18] However, the recent finding that asthma risk is increased similarly with attendance at outdoor pools demonstrates that trichloramine cannot be the main cause of respiratory effects in swimmers, because this highly volatile gas is quickly dispersed into the atmosphere when it is released outdoors.[19] Substances causing asthma and breathing difficulties in swimmers must be sought among the chlorination products present in pool water or floating at the surface of the pool as aerosols or vapors. If this reasoning is correct, then it means that the respiratory impact of chlorinated pools can be assessed correctly only by taking into account total exposure to chlorinated pools, regardless of the type of pool and the conditions of attendance.

The aim of the present study was to assess for the first time the overall impact of chlorinated pool exposure on the respiratory health of adolescents, by considering the total time spent in indoor or outdoor chlorinated pools. The study took advantage of the existence in Belgium of copper-silver pools, which enabled us to identify a reference population of swimmers with no or minimal exposure to chlorination products.

METHODS

The ethics committee of the Faculty of Medicine of the Catholic University of Louvain approved the study protocol. We recruited adolescents in 3 secondary schools in the southern part of Belgium, in the cities of Louvain-la-Neuve, Bastogne, and Lessines. Participation rates were similar among the 3 schools, as well as between girls and boys (between 70.6% and 72.1%).

Students in Louvain-la-Neuve had access to an indoor pool sanitized through the copper-silver method, whereas students at the other 2 schools could visit only indoor pools disinfected with chlorine. According to the Belgian legislation, each swimming pool is required to check the microbial and chemical qualities of water regularly by measuring several parameters, including active (0.5–1.5 ppm) and combined (<2 ppm) chlorine. In 2003, the legislation was enforced through a lowering of the standard for combined chlorine (<0.8 ppm) and the setting of a standard for trichloramine levels in pool air (<500 $\mu g/m^3$ in air sampled 1.5 m above the pool surface). The concentrations of trichloramine in air ranged from 300 to 500 $\mu g/m^3$. There were occasional exceedances of active or combined chlorine levels but without attaining levels that might pose a risk to swimmers, according to international guidelines (concentrations of active and combined chlorine never exceeded 4 ppm). The water of the copper-silver pool was sanitized with concentrations of copper (0.6–1.2 mg/L) and silver (2–10 $\mu g/L$) that were lower than drinking water guidelines.[20]

The protocol for examining students was described in detail elsewhere.[19] Briefly, parents completed a questionnaire inquiring about the health of their child and risk factors for asthma and allergic diseases. The questions

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about respiratory symptoms and allergic diseases were those of the International Study of Asthma and Allergy in Childhood.[21] The questionnaire also included questions intended to estimate the total time the child had spent in indoor or outdoor chlorinated pools. Students were asked not to visit a chlorinated pool during the 48 hours preceding the study. The examination of students, which was performed in schools, included measurements of height and body weight, an interview with the adolescent about respiratory symptoms, collection of a blood sample, and screening for exercise-induced bronchoconstriction (EIB). The EIB test consisted of measuring the decrease in forced expiratory volume in 1 second (FEV_1) after 6 minutes of indoor running with submaximal effort, and results were considered positive when the exercise caused a decrease in FEV_1 of $\geq$10%.[22] Asthma medication use was not discontinued before the test. Asthma was defined either as "ever asthma," corresponding to asthma diagnosed at any time by a physician, or as "current asthma," corresponding to physician-diagnosed asthma that, at the time of the study, was being treated with medication (bronchodilators and/or inhaled corticosteroids) and/or was associated with positive EIB test results. Because the examination of adolescents in schools precluded any allergic provocation test, we screened for allergies by measuring total and aeroallergen-specific immunoglobulin E (IgE) concentrations in serum, using the Immulite IgE kit (Diagnostic Products, Los Angeles, CA). Sensitization against specific aeroallergens was defined as serum concentrations of specific IgE of >0.35 kIU/L.

We assessed associations between outcomes and cumulative chlorinated pool attendance (CPA) stratified into 4 categories, that is, <100 hours, 100 to 500 hours, 500 to 1000 hours, or >1000 hours. Adjusted odds ratios (ORs) for the outcomes in these categories were calculated with backward logistic regression models, using as the reference level the occurrence of the outcome among adolescents with CPA of <100 hours. Backward selection started with a model including all potential control variables and each step was performed by deleting the least-significant predictor, until the model contained only variables with $P < .20$. We tested a total of 26 potential predictors, including CPA, gender, total and aeroallergen-specific IgE levels, parental asthma or allergy, and maternal smoking during pregnancy. Population attributable risks (PARs) were calculated by using the formula $P(OR - 1)/[P(OR - 1) + 1]$, where P is the prevalence of the exposure and OR is the adjusted OR attributable to the exposure. The level of statistical significance was set at $P < .05$.

RESULTS

Table 1 shows the characteristics of participants. The 2 genders were similarly represented in the 3 schools except for the school in Bastogne, which included more girls. There were no or little differences between the 3 schools with respect to age (15 years, on average), ethnicity, and indicators of respiratory health except for the prevalence of hay fever and FEV_1 values, which were higher in Bastogne and Lessines, respectively. Students in Louvain-la-Neuve had higher socioeconomic status than their peers, as reflected by parental education levels and several lifestyle factors, such as breastfeeding, exposure to tobacco smoke, and access to a backyard pool. Because they had access to an indoor copper-silver pool, students in Louvain- la-Neuve had much lower CPA levels than did those in Bastogne and Lessines. Among them, 118 had a lifetime CPA value of <100 hours. Those students were selected to constitute the reference group.

We first assessed associations between chlorinated pools and outcomes through stratification of data into categories of increasing CPA (Table 2). The rate of sensitization to major aeroallergens did not vary with CPA, whereas the total serum IgE concentrations showed, if anything, a tendency to decrease. There also was little variation in FEV_1 values, which showed only a very modest increase (2%, on average) between the lowest and highest CPA categories. Prevalences of wheezing and EIB, although increased in all groups with CPA values of >100 hours, did not show any significant exposure-related trend. In contrast, the prevalences and odds of ever asthma, current asthma, cough, and shortness of breath increased almost linearly with the time spent in chlorinated pools. These exposure-related increases persisted when students in Bastogne and Lessines, who visited only chlorinated pools, and those in Louvain-la-Neuve, who also had access to the copper-silver pool, were examined separately (eg, P values for trend were .02 and .07 for ever asthma and .02 and .04 for current asthma, respectively).

For cough and shortness of breath, these associations even persisted when adolescents with a diagnosis of asthma were excluded (P for trend of .004 and .05, respectively). The risk of hay fever was increased in all groups with CPA values of >100 hours, whereas the risk of allergic rhinitis was increased only in the group with the highest CPA value (>1000 hours).

Risks of respiratory symptoms (Table 3) and asthma (Table 4) associated with CPA were strongly influenced by atopic status. CPA increased the risks of wheezing, cough, shortness of breath, ever asthma, and current asthma only among adolescents with

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TABLE 1 Characteristics of Adolescents

Characteristics	Louvain-la-Neuve ($N = 357$)	Bastogne ($N = 349$)	Lessines ($N = 141$)	*P*
Adolescents				
Boys, *n* (%)	167 (46.8)	130 (37.2)	72 (51.1)	.006
Age, mean ± SD, y	15.4 ± 0.81	15.5 ± 0.83	15.5 ± 0.87	.54
White, *n* (%)	343 (96.1)	344 (98.6)	137 (97.2)	.13
BMI, mean ± SD, kg/m^2	20.1 ± 2.3	21.0 ± 3.3	21.0 ± 3.2	<.001
Parents				
Asthma, *n* (%)	49 (13.7)	39 (11.2)	25 (17.7)	.15
Hay fever, *n* (%)	101 (28.3)	71 (20.3)	36 (25.5)	.05
Allergic rhinitis, *n* (%)	60 (16.8)	52 (14.9)	21 (14.9)	.75
Education, *n* (%)	278 (77.9)	98 (28.1)	28 (19.9)	<.001
Early life				
Birth weight, mean ± SD, kg	3.32 ± 0.55	3.33 ± 0.58	3.25 ± 0.61	.35
Breastfeeding, *n* (%)	305 (85.4)	191 (54.7)	76 (53.9)	<.001
Child care attendance, *n* (%)	241 (67.5)	111 (31.8)	43 (30.5)	<.001
Exposure to tobacco smoke				
Active smoking, *n* (%)	20 (5.6)	14 (4.0)	16 (11.3)	.007
Smoking during pregnancy, *n* (%)	25 (7.0)	55 (15.8)	25 (17.7)	<.001
Passive smoking at home, *n* (%)	20 (5.6)	14 (4.0)	16 (11.3)	.007
Environment				
No. of older siblings, mean ± SD	0.93 ± 0.98	0.98 ± 1.02	0.85 ± 0.99	.43
Mold on bedroom walls, *n* (%)	30 (8.4)	19 (5.4)	10 (7.1)	.30
Housecleaning with bleach, *n* (%)	77 (21.6)	91 (26.1)	60 (42.6)	<.001
Living close to busy road, *n* (%)	55 (15.4)	56 (16.0)	43 (30.5)	<.001
Exposure to pets since birth, *n* (%)	51 (14.4)	47 (13.5)	17 (12.1)	.79
Swimming pool attendance				
Indoor				
n (%)	243 (68.1)	348 (99.7)	138 (97.9)	<.001
CPA, median (IQR), h	126 (48–286)	400 (255–657)	407 (217–724)	<.001
Outdoor				
n (%)	270 (75.6)	183 (52.4)	61 (43.3)	<.001
CPA, median (IQR), h	229 (70–477)	147 (57–336)	308 (134–599)	<.001
Indoor and outdoor				
n (%)	324 (90.8)	348 (99.7)	138 (97.9)	<.001
CPA, median (IQR), h	312 (126–649)	524 (300–895)	522 (280–1002)	<.001
Aeroallergen-specific serum IgE, *n* (%)				
Dust mite	103 (28.9)	103 (28.9)	32 (22.7)	.34
Dog	15 (4.2)	15 (4.2)	10 (7.1)	.40
Cat	49 (13.7)	49 (13.7)	14 (9.9)	.50
Pollen	65 (18.2)	65 (18.2)	19 (13.5)	.002
Mold	11 (3.1)	11 (3.1)	7 (5.0)	.14
≥1 aeroallergen	136 (38.1)	136 (38.1)	43 (30.5)	.19
Total serum IgE level, median (IQR), kIU/L	60.0 (22.1–173)	60.0 (22.1–173)	44.9 (13.8–147)	.004
Respiratory function and diseases				
Proportion of predicted FEV_1, mean ± SD, %	99.0 ± 13.9	100.9 ± 13.9	104.0 ± 15.1	.001
Positive EIB test results, *n* (%)	51 (14.3)	63 (18.0)	22 (14.6)	.39
Ever asthma, *n* (%)	38 (10.6)	36 (10.3)	14 (9.9)	.97
Current asthma, *n* (%)[a]	21 (5.9)	21 (6.0)	10 (7.1)	.87
Hay fever, *n* (%)	45 (12.6)	67 (19.2)	19 (13.5)	.04
Allergic rhinitis, *n* (%)	60 (16.8)	58 (16.6)	23 (16.3)	.99

P values indicate the level of statistical significance in $[\chi]^2$ tests (prevalences) or Kruskal-Wallis analyses of variance (mean or median values). IQR indicates interquartile range.

[a] Includes 52 subjects with ever physician-diagnosed asthma who, at the time of the study, were receiving asthma medication ($n = 37$) and/or had positive EIB test results ($n = 24$).

higher total serum IgE levels and had no influence on these outcomes among the other adolescents. For the risks of ever or current asthma, the same patterns of interactions between CPA and atopy, defined as sensitization to any aeroallergen, emerged. An interaction with the risk of EIB also emerged and was significantly increased only among sensitized adolescents. For respiratory symptoms, however, the interaction between CPA and aeroallergen sensitization was less clear cut, with CPA increasing the risk of cough for adolescents without and with atopy and the risk of shortness of breath only for adolescents without atopy (Table 4).

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TABLE 2 Total and Any Aeroallergen-Specific Serum IgE Levels, FEV_1, and Risks of Respiratory Symptoms, EIB, and Physician-Diagnosed Allergic Diseases Among Adolescents With Increasing Cumulative CPA

	CPA				*P*
	<100 h	100–500 h	500–1000 h	>1000 h	
N	114	369	221	143	
CPA, median (IQR), h	24 (0–56)	288 (194–378)	693 (600–825)	1483 (1165–1967)	
Boys, *n* (%)	63 (55.3)	147 (39.8)	99 (44.8)	60 (42.0)	.03
Age, mean ± SD, y	15.5 ± 0.88	15.5 ± 0.84	15.5 ± 0.85	15.5 ± 0.86	.88
Total serum IgE level, median (IQR), kIU/L	58.6 (22.8–152)	53.1 (19.8–157)	42.3 (16.1–126)	44.9 (15.4–149)	.10
Aeroallergen-specific serum IgE, *n* (%)	44 (38.6)	142 (38.5)	62 (37.1)	53 (37.0)	.90
Proportion of predicted FEV_1, mean ± SD, %	100.2 ± 14.7	99.7 ± 14.0	100.9 ± 14.4	103.1 ± 13.8	.11
Wheezing					
n (%)	9 (8.8)	46 (12.5)	24 (10.9)	23 (16.1)	.12
OR (95% CI)[a]	1.00 (1.00–1.00)	1.40 (0.65–3.00)	1.32 (0.58–2.00)	2.09 (0.90–4.84)	
Cough					
n (%)	11 (9.6)	62 (16.8)	32 (14.5)	39 (27.3)	<.001
OR (95% CI)[b]	1.00 (1.00–1.00)	1.39 (0.68–2.85)	1.32 (0.62–2.82)	3.00 (1.41–6.38)	
Chest tightness					
n (%)	3 (2.6)	16 (4.3)	7 (3.2)	10 (7.0)	.37
OR (95% CI)[c]	1.00 (1.00–1.00)	1.25 (0.34–4.52)	1.02 (0.25–4.17)	2.78 (0.72–10.7)	
Shortness of breath					
n (%)	4 (3.5)	23 (6.2)	17 (7.7)	16 (11.2)	.01
OR (95% CI)[d]	1.00 (1.00–1.00)	1.49 (0.49–4.55)	2.15 (0.68–6.78)	3.25 (1.01–10.5)	
Positive EIB test results					
n (%)	13 (11.4)	66 (17.9)	33 (14.9)	24 (16.8)	.68
OR (95% CI)[e]	1.00 (1.00–1.00)	1.61 (0.84–3.11)	1.28 (0.64–2.61)	1.56 (0.74–3.28)	
Ever asthma					
n (%)	6 (5.3)	36 (9.8)	23 (10.4)	23 (16.1)	.007
OR (95% CI)[f]	1.00 (1.00–1.00)	1.80 (0.71–4.57)	2.08 (0.79–5.48)	3.74 (1.40–9.93)	
Current asthma					
n (%)	2 (1.8)	22 (6.0)	14 (6.4)	17 (11.9)	.002
OR (95% CI)[g]	1.00 (1.00–1.00)	2.99 (0.66–13.5)	3.77 (0.81–17.5)	8.05 (1.74–37.2)	
Hay fever					
n (%)	8 (7.0)	63 (17.1)	35 (15.9)	25 (17.4)	.10
OR (95% CI)[h]	1.00 (1.00–1.00)	3.04 (1.34–6.87)	2.64 (1.12–6.23)	3.49 (1.42–8.57)	
Allergic rhinitis					
n (%)	12 (10.5)	65 (17.9)	38 (17.3)	26 (18.2)	.21
OR (95% CI)[i]	1.00 (1.00–1.00)	2.00 (0.99–4.03)	1.96 (0.93–4.11)	2.38 (1.08–5.22)	

P values indicate the level of statistical significance in χ^2 tests for trend. CI indicates confidence interval; IQR, interquartile range.

[a] Adjusted for parental allergy, total serum IgE level, BMI, birth weight, maternal smoking during pregnancy, active smoking, pacifier use, house with double glazing, and living <100 m from a busy road.

[b] Adjusted for parental asthma, gender, older siblings, parental smoking, air fresheners, house with double glazing, and living <100 m from a busy road.

[c] Adjusted for gender, birth weight, pacifier use, smoking, mold on bedroom walls, and living <100 m from a busy road.

[d] Adjusted for gender, maternal smoking during pregnancy, breastfeeding, smoking, air fresheners, mold on bedroom walls, and living <100 m from a busy road.

[e] Adjusted for parental asthma, gender, total serum IgE level, sensitization to any aeroallergen, maternal smoking during pregnancy, older siblings, house cleaning with bleach, and mold on bedroom walls.

[f] Adjusted for sensitization to any aeroallergen, parental smoking, birth weight, breastfeeding, pacifier use, and living <100 m from a busy road.

[g] Adjusted for parental asthma, gender, sensitization to any aeroallergen, maternal smoking during pregnancy, and house cleaning with bleach.

[h] Adjusted for parental allergy, BMI, sensitization to any aeroallergen, day care attendance, and air fresheners.

[i] Adjusted for parental allergy, BMI, total serum IgE level, sensitization to any aeroallergen, birth weight, and maternal smoking during pregnancy.

CPA also interacted with aeroallergen sensitization, and specifically pollen and dust mite sensitization, to increase the risks of hay fever and allergic rhinitis (Table 5). The exposure-response relationships for hay fever and allergic rhinitis were noticeably different, and they also were different from the relationships observed for asthma. Although the odds for allergic rhinitis were increased only among sensitized adolescents with the highest CPA values (>1000 hours), the odds for hay fever were increased significantly among sensitized adolescents with CPA values of >100 hours and above this threshold tended to plateau. This nonlinear relationship suggests an early increase of hay fever risk toward a plateau that seems to be reached when CPA exceeds 500 hours. To test this hypothesis, we further assessed the dose-response relationships by stratifying data for adolescents with CPA values of <500 hours. As shown in Fig 1, the prevalence of hay fever among these adolescents increased linearly with CPA, whether all subjects were considered or boys and girls were considered separately, and

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TABLE 3 Risks of Wheezing, Cough, and Shortness of Breath With Increasing CPA for Adolescents Classified as Nonatopic or Atopic on the Basis of Total or Aeroallergen-Specific Serum IgE Levels

Symptom and CPA	Total IgE Level		Aeroallergen-Specific IgE Level	
	<30 kIU/L (*N* = 315)	>30 kIU/L (*N* = 532)	<0.35 kIU/L (*N* = 526)	>0.35 kIU/L (*N* = 321)
Wheezing				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	0.95 (028–3.32)	2.00 (0.72–5.55)	1.10 (0.39–3.11)	1.95 (0.60–6.37)
500–1000 h	0.89 (0.25–3.20)	1.73 (0.57–5.24)	1.16 (0.39–3.50)	1.52 (0.93–5.37)
>1000 h	1.04 (0.27–4.10)	3.36 (1.11–10.2)	1.49 (0.48–4.65)	3.45 (0.96–12.4)
P for trend	.81	.07	.31	.21
Cough				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	0.76 (0.27–2.16)	2.09 (0.75–5.79)	1.09 (0.48–2.48)	2.74 (0.58–13.0)
500–1000 h	0.49 (0.16–1.52)	2.89 (0.97–8.18)	0.90 (0.37–2.20)	3.61 (0.73–18.0)
>1000 h	2.19 (0.74–6.50)	3.89 (1.31–11.6)	2.40 (1.00–5.78)	5.65 (1.12–28.6)
P for trend	.07	.008	.03	.02
Shortness of breath				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	0.86 (0.19–3.86)	3.38 (0.42–27.0)	0.90 (0.23–3.55)	3.32 (0.39–28.4)
500–1000 h	1.03 (0.21–4.99)	5.25 (0.64–43.3)	1.34 (0.33–5.49)	4.85 (0.55–43.0)
>1000 h	1.10 (0.21–5.63)	9.58 (1.16–79.3)	2.15 (0.52–8.93)	6.03 (0.64–58.8)
P for trend	.65	.005	.04	.13

Numbers in the 4 CPA categories were as follows: IgE level of <30 kIU/L, 35, 128, 95, and 57; IgE level of >30 kIU/L, 79, 241, 126, and 86; level of IgE against any aeroallergen of <0.35 kIU/L, 70, 227, 139, and 90; level of IgE against any aeroallergen of >0.35 kIU/L, 43, 142, 82, and 53. ORs were adjusted for the same predictors as in Table 2. *P* values indicate the level of statistical significance in χ^2 tests for trend. CI indicates confidence interval.

TABLE 4 Risks of Ever Asthma, Current Asthma, and EIB With Increasing CPA for Adolescents Classified as Nonatopic or Atopic on the Basis of Total or Aeroallergen-Specific Serum IgE Levels

	Total IgE Level		Aeroallergen-Specific IgE Level	
	<30 kIU/L (*N* = 315)	>30 kIU/L (*N* = 532)	<0.35 kIU/L (*N* = 526)	>0.35 kIU/L (*N* = 321)
Ever asthma				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	0.62 (0.15–2.64)	3.14 (0.88–11.2)	1.05 (0.27–3.98)	2.77 (0.76–10.1)
500–1000 h	0.81 (0.18–3.62)	3.75 (1.01–13.9)	1.27 (0.32–5.08)	2.88 (0.75–11.0)
>1000 h	0.60 (0.11–3.35)	7.82 (2.11–29.1)	1.42 (0.33–6.09)	7.11 (1.83–27.5)
P for trend	.70	.0004	.44	.003
Current asthma				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	0.55 (0.05–6.59)	5.44 (0.69–43.0)	0.92 (0.10–8.81)	5.39 (0.67–43.1)
500–1000 h	0.39 (0.02–6.68)	8.19 (1.02–65.8)	0.79 (0.07–9.29)	7.32 (0.80–59.6)
>1000 h	1.33 (0.11–16.8)	14.9 (1.85–120)	3.02 (0.33–28.0)	12.6 (1.52–105)
P for trend	.76	.0005	.09	.006
Positive EIB test results				
OR (95% CI)				
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	2.32 (0.67–7.99)	1.46 (0.66–3.23)	0.95 (0.43–2.10)	4.19 (1.18–14.9)
500–1000 h	0.95 (0.25–3.52)	1.38 (0.59–3.26)	0.66 (0.27–1.60)	3.83 (1.04–14.1)
>1000 h	1.42 (0.35–5.80)	1.71 (0.70–4.20)	1.03 (0.42–2.58)	3.67 (0.93–14.6)
P for trend	.49	.24	.71	.26

Numbers in the 4 CPA categories stratified according to total serum IgE levels or aeroallergen-specific serum IgE levels were the same as in Table 3. ORs were adjusted for the same predictors as in Table 2. *P* values indicate the level of statistical significance in χ^2 tests for trend. CI indicates confidence interval.

even when only adolescents without parental hay fever were considered. Quite remarkably, this early increase was found only among subjects who were sensitized to aeroallergens or pollen.

The PARs for atopic diseases associated with CPA were calculated by considering as exposed the students with atopy who had spent >100 hours in chlorinated swimming pools. When atopy was defined on the basis of total serum IgE levels, these calculations yielded PARs of 63.4% for ever asthma, 79.2% for current asthma, 62.1% for hay fever, and 35.0% for allergic rhinitis. We obtained similar PAR estimates when atopy was defined as sensitization to any aeroallergen (ever asthma: 46.2%; current asthma: 67.3%; hay fever: 49.8%; allergic rhinitis: 29.6%). By comparison, the PARs for ever and current asthma attributable to maternal smoking during pregnancy, the only other lifestyle risk factor remaining in the model with *P* values of <.20 ($P = .09$ and $P = .11$, respectively), were 8.3% and 10.1%, respectively.

DISCUSSION

Our findings show that CPA during childhood interacts with atopic status to increase the risk of asthma, hay fever, and allergic rhinitis. Although one could evoke the possibility of reverse causation to explain the associations between asthma and indoor pool attendance on the basis that indoor swimming is recommended frequently for individuals with asthma, this explanation does not hold for associations with hay fever and allergic rhinitis or for associations with respiratory symptoms (cough and shortness of breath) observed in the absence of a diagnosis of asthma. The hypothesis of reverse causation seems even more unlikely because it implies that only individuals with atopy would be encouraged to swim, a possibility refuted by

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TABLE 5 Risks of Hay Fever and Allergic Rhinitis With Increasing CPA for Adolescents Classified as Nonatopic or Atopic on the Basis of Total or Aeroallergen-Specific Serum IgE Levels

	Total IgE Level		Aeroallergen-Specific IgE Level		Pollen-Specific IgE Level		Dermatophagoides-Specific IgE Level	
	<30 kIU/L (*N* = 315)	>30 kIU/L (*N* = 532)	<0.35 kIU/L (*N* = 526)	>0.35 kIU/L (*N* = 321)	<0.35 kIU/L (*N* = 671)	>0.35 kIU/L (*N* = 176)	<0.35 kIU/L (*N* = 622)	>0.35 kIU/L (*N* = 225)
Hay fever								
OR (95% CI)								
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)		
100–500 h	0.61 (0.11–3.27)	4.50 (1.73–11.7)	1.31 (0.36–4.80)	4.68 (1.66–13.2)	2.02 (0.66–6.14)	4.13 (1.22–14.0)		
500–1000 h	0.66 (0.12–3.69)	3.72 (1.36–10.2)	1.46 (0.38–5.69)	3.27 (1.10–9.72)	1.79 (0.55–5.76)	4.38 (1.18–16.2)		
>1000 h	0.52 (0.08–3.55)	5.39 (1.89–15.4)	1.36 (0.32–5.81)	5.70 (1.82–17.9)	2.30 (0.69–7.70)	6.56 (1.61–26.9)		
P for trend	.99	.02	.65	.049	.30	.03		
Allergic rhinitis								
OR (95% CI)								
<100 h	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)			1.00 (1.00–1.00)	1.00 (1.00–1.00)
100–500 h	4.31 (0.53–35.1)	1.88 (0.87–4.03)	1.70 (0.56–5.21)	2.04 (0.83–5.04)			1.98 (0.78–5.01)	2.00 (0.69–5.78)
500–1000 h	3.52 (0.41–30.0)	2.01 (0.88–4.59)	1.63 (0.50–5.28)	1.93 (0.74–5.05)			1.48 (0.55–3.97)	2.65 (0.84–8.31)
>1000 h	3.54 (0.39–32.5)	2.22 (0.93–5.31)	1.44 (0.39–5.23)	3.54 (1.27–9.84)			1.78 (0.62–4.90)	3.46 (1.04–11.5)
P for trend	.65	.11	.84	.10			.66	.012

Numbers in the 4 CPA categories stratified according to total serum IgE or aeroallergen-specific serum IgE levels were the same as in Table 3. Numbers in the other CPA categories were as follows: pollen-specific IgE level of <0.35 kIU/L, 92, 283, 180, and 116; pollen-specific IgE level of >0.35 kIU/L, 22, 86, 41, and 27; Dermatophagoides-specific IgE level of <0.35 kIU/L, 83, 267, 169, and 103; level of IgE against any aeroallergen of >0.35 kIU/L, 31, 102, 52, and 40. ORs were adjusted for the same predictors as in Table 2. *P* values indicate the level of statistical significance in χ^2 tests for trend. CI indicates confidence interval.

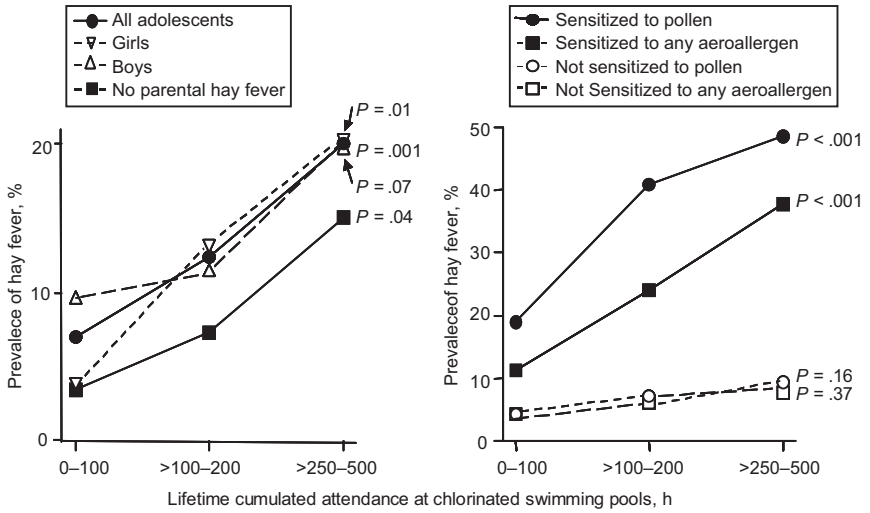


FIGURE 1
Prevalences of hay fever among adolescents according to their attendance at indoor or outdoor chlorinated swimming pools, for all subjects, for girls and boys separately, for adolescents without parental hay fever, and for adolescents sensitized or not sensitized against pollen or any aeroallergen. *P* values correspond to χ^2 tests for trend.

the fact that the proportion of individuals with atopy showed a tendency to decrease with lifetime pool attendance. These associations cannot be ascribed to swimming itself, because none of the studied outcomes was influenced by attendance at the copper-silver pool. The only plausible explanation is that the chlorine-based oxidants in water or air floating at the pool surface cause some airway changes and promote the development of allergic diseases.

The present study confirms the pool chlorine-atopy interaction in asthma development that we found previously among schoolchildren who attended indoor chlorinated pools.[11] Although they were based on different populations of swimmers, 2 Scandinavian studies reported observations that also suggest the existence of interactive effects of atopy and exposure to chlorinated pools. Among competitive swimmers, Helenius et al[6] found that asthma risk was >10 times greater among elite swimmers who had atopy than among those who did not. Investigating the effects of infant swimming on the respiratory health of children, Nystad et al[23] reported that infant swimming increased the risk of wheezing among children whose parents had atopy but not among those whose parents did not. Because children of parents with atopy are more likely to have atopy themselves, this study points indirectly to an interaction between atopic status and CPA in the development of childhood asthma.

Probably the most important finding of our study is that the pool chlorine-atopy interaction described initially for asthma may extend to other common allergic diseases, such as hay fever and allergic rhinitis. Exposure-response relationships seemed to

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differ according to the type of respiratory allergy, which is not surprising, given the variable deposition of inhalants along the respiratory tract. In particular, the interaction between chlorine and atopy in the risk of hay fever was triggered by much lower CPA levels, compared with asthma and allergic rhinitis. These differences in exposure-response relationships, as well as probably some differences in chlorine levels in swimming pools, might explain some conflicting results from other studies. In a retrospective analysis of data on medical history and swimming pool attendance of a cohort of adults in Germany, Kohlhammer et al[16] found that early school pool attendance was associated with increased risk of hay fever but no association with asthma emerged, in contrast to our findings. We think that the explanation for this discrepancy might lie in the fact that the German cohort was composed of adults who, during their childhood, had been much less exposed to chlorinated pools than are present-day children. It is possible that, when they were children, these adults had attended chlorinated pools enough to exceed the CPA threshold for inducing hay fever but not enough to attain the CPA threshold for inducing asthma. More recently, the same German team further explored the relationship between swimming during infancy and the development of allergies by analyzing 6-year follow-up data for a prospective birth cohort study.[24] The authors could not detect clear associations between swimming and the development of allergic diseases. The authors explained the findings on the basis of the fact that the German chlorine standards at the time those children attended pools had already been decreased to levels that were much lower (by factors of 2 and 10, respectively) than the standards in effect in the former German study[24] and our studies.[10–12] Another possible explanation would be that the population studied by Schoefer et al[24] was still too young for detection of associations with asthma and other atopic diseases, conditions that usually are diagnosed correctly later in childhood.[25]

Disruption of epithelial barriers increasingly seems to be a basic mechanism in allergic sensitization.[26] Most potent allergens, such as dust mites, cat dander, and pollen, display a proteolytic activity that allows then to open tight junctions, to cross epithelial barriers more easily, and from there to interact with immune cells.[27–29] Chlorination products such as hypochlorous acid and chloramines are membrane-permeant oxidants that also can breach tight junctions and can facilitate the transepithelial delivery of allergens. These chemicals might thus promote allergic diseases through a mechanism similar to allergens displaying proteolytic activity. Because there is no reason to think that chlorine-based oxidants would react differently with the epithelia of the upper and lower airways, we think that differences in exposure-response relationships between hay fever and asthma might reflect primarily differences in the doses of chlorination products deposited along the respiratory tract. If the risk of hay fever increases significantly with much lower CPA levels than asthma, then it might be simply because the upper airways receive most of the burden of chlorine that swimmers inhale actively at the surface of the pool.[30]

CONCLUSIONS

Our study shows that CPA exerts a strong adjuvant effect on the development of asthma, hay fever, and allergic rhinitis. These findings reinforce the need to pursue research in this area and to enforce regulations concerning the levels of these chemicals in water and air of swimming pools.

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Impact of Chlorinated Swimming Pool Attendance on the Respiratory Health of Adolescents

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Infant swimming in chlorinated pools and the risks of bronchiolitis, asthma and allergy

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ABSTRACT: Recent studies suggest that swimming in chlorinated pools during infancy may increase the risks of lower respiratory tract infection. The aim of the present study was to assess the influence of swimming in chlorinated pools on the risks of bronchiolitis and its late consequences.

A total of 430 children (47% female; mean age 5.7 yrs) in 30 kindergartens were examined. Parents completed a questionnaire regarding the child's health history, swimming practice and potential confounders.

Attendance at indoor or outdoor chlorinated pools ever before the age of 2 yrs was associated with an increased risk of bronchiolitis (OR 1.68; 95% CI 1.08–2.68; p=0.03), which was exposure-dependent for both types of pool (p-value for trend <0.01). Associations persisted, and were even strengthened, by the exclusion of other risk factors. Among children with no parental antecedents of atopic disease or no day-care attendance, odds ratios for bronchiolitis amounted to 4.45 (1.82–10.9; p=0.001) and 4.44 (1.88–10.5; p=0.007) after >20 h spent in chlorinated pools during infancy. Infant swimmers who developed bronchiolitis also showed higher risks of asthma and respiratory allergies later in childhood.

Swimming pool attendance during infancy is associated with a higher risk of bronchiolitis, with ensuing increased risks of asthma and allergic sensitisation.

KEYWORDS: Bronchiolitis, infant swimming, respiratory infection, swimming pool

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Bronchiolitis is an acute infection of the small airways that primarily affects young infants, most often those aged 2–24 months. The main causative agent is respiratory syncytial virus (RSV), although other viruses may sometimes be involved. The disease occurs with a seasonal pattern, peaking during winter in temperate climates and the rainy season in tropical climates [1, 2]. The disease burden of bronchiolitis is substantial and seems to have increased in most developed countries since the 1960s–1970s [3]. In the USA and Europe, the annual incidence of bronchiolitis during the first year of life is estimated to be 10–20%, but incidences as high as 30% have been reported in some urban areas [3, 4]. Since bronchiolitis increases the risk of childhood asthma [5, 6], it also contributes to the rising incidence of chronic respiratory diseases in children.

Factors that may increase the frequency or severity of bronchiolitis are male sex, low gestational age, young or unmarried mothers, infant condition at birth (Apgar score), lack of breastfeeding, early weaning, congenital heart or lung diseases, and a family history of atopic diseases. The risk of bronchiolitis is also higher for infants living with older siblings, attending a day-care centre or exposed to environmental tobacco smoke [2, 7]. Since ambient air pollution appears to play, if any, a marginal role [8], factors driving the rise of the disease in industrialised countries remain largely unknown.

Infant swimming is a practice that has been greatly popularised in most industrialised countries. NYSTAD and co-workers [9, 10] were among the first to draw attention to the risks that this practice may pose to the airways of infants. These authors noted that infants of atopic parents were more likely to develop wheezing or lower respiratory tract infections when they were attending swimming pools. In a cross-sectional study of schoolchildren aged 10–13 yrs, it was also found that infant swimmers were more likely to develop asthma and recurrent bronchitis than their peers who had never attended a chlorinated pool before the age of 2 yrs [11, 12]. Interestingly, this poorer respiratory health of infant swimmers was associated with lower serum levels of Clara cell protein (CC16), a protein protecting from inflammation in acute RSV infection [13]. These findings led to the postulate that the chlorine used to disinfect swimming pools can cause airway

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changes making the lungs more sensitive to infection- and asthma-inducing agents [12]. A Swedish study also described decreased levels of serum CC16 in children who swam during infancy compared to those who did not [14], providing further evidence that terminal airways and, in particular, Clara cells may be damaged during infant swimming.

Given these associations between infant swimming, serum CC16 and RSV infection, it was hypothesised that the infant swimming practice should be associated with a higher risk of bronchiolitis. In order to test this hypothesis, the associations between infant swimming, bronchiolitis and its sequelae were explored in a cross-sectional study focused on young schoolchildren. Since both outdoor and indoor pools may affect the airways of swimmers [15], the present study was not limited to baby swimming lessons in indoor pools, but considered all kinds of swimming activity during infancy, regardless of the type of chlorinated pool and the conditions of attendance.

METHODS

Study population

The study participants were 5–6-yr-old children from 30 schools located mainly in the areas of Brussels and Liège (Belgium). These children were recruited in the framework of a prospective study on the respiratory impact of air pollution. A questionnaire and an informed consent document were distributed to children in the third year of kindergarten. Of the 839 children who received these documents, 430 returned the questionnaire with the informed consent document filled in by the parents. The overall participation rate was 51.3%, but showed great variation between schools (20–90%). Since the participation rate did not correlate with the prevalence of the main outcomes and risk factors, it was decided to retain all schools for the statistical analysis and to add the participation rate to the list of potential confounders. The ethics committee of the Faculty of Medicine of the Catholic University of Louvain (Louvain, Belgium) approved the study protocol, which complied with all applicable requirements of the international regulations.

Protocol

Parents completed a detailed questionnaire inquiring about the health of their child, respiratory symptoms, family antecedents, care during early life and all lifestyle or environmental factors known or suspected to influence the risk of bronchiolitis. Questions regarding respiratory symptoms were those of the International Study of Asthma and Allergies in Childhood [16]. Wheezing was identified by a positive answer to the question ''Has your child had episodes of wheezing during the last 12 months?''. The questionnaire included specific questions asking whether the child had ever been diagnosed for most common childhood diseases. Among these, there were seven distinct questions about the following respiratory diseases: asthma, bronchitis, bronchiolitis, pneumonia (or bronchopneumonia), hay fever, allergic rhinitis, and sinusitis. For each question, the parents were asked to reply ''yes'' or ''no''. In the case of a positive reply, parents were then asked to indicate the exact age at which the disease was diagnosed. The questionnaire also comprised questions about sport and recreational activities. For swimming practice, parents were asked to specify the type of pool attended by their child, the type of disinfection method used (even though almost all of them use chlorine), the frequency of attendance and the age at which their child started to attend the pool regularly. This information served to calculate the cumulative pool attendance (CPA) at indoor or outdoor chlorinated pools, separately or combined, before the age of 2 yrs (during infancy) or later in life (during childhood). Children were examined in schools during 09:00–16:00 h. Height and body weight were measured. Since the examination of children in schools precluded any provocation test, screening for respiratory allergies in school was performed noninvasively by measuring specific immunoglobulin E in nasal mucosa using the Rhinostick test (made by F. Marcucci) [17]. This test was successfully performed in 372 children, in whom the following allergens were screened for: cat epithelium, *Dermatophagoides pteronyssinus*, *Anthoxanthum odoratum*, *Parietaria officinalis* and a mix of tree allergens containing *Betula odorata*, *Corylus avellana*, *Carpinus betulus* and *Alnus incana*. Regarding sport and recreational activities organised by the schools, the school directors were asked to complete a questionnaire that permitted the information given by the parents to be cross-checked and completed.

Swimming pools

In Belgium, every public swimming pool is legally required to regularly check the microbial and chemical quality of the water by measuring several parameters, including active (0.5–1.5 ppm) and combined chlorine (<2 ppm). In 2003, the legislation was strengthened by a lowering of the standard for combined chlorine (<0.8 ppm) and the setting of a standard for trichloramine in pool air (<500 $\mu g \cdot m^{-3}$ in air sampled 1.5 m above the pool surface). Since there were no public outdoor pools in the areas studied, the open-air swimming pools attended by infant swimmers were mostly residential. In Belgium, as in most countries, there are no specific regulations for these privately owned swimming pools, which are disinfected according to the instructions of the chlorine supplier (active chlorine 1–2 ppm).

Statistical analysis

Continuous variables were described using the median with interquartile range. Differences between infant swimmers and their controls were assessed using the two-sided Mann–Whitney U-test for the CPA indices and an unpaired t-test for the other variables. Binary variables were compared using the Chi-squared test, or with a Chi-squared test for trend for the analysis of exposure–response relationships. Logistic regression models were used to assess the associations between swimming and bronchiolitis while adjusting for potential confounders. CPA at outdoor or indoor pools before the age of 2 yrs was categorised as never, >0–20 h and >20 h. Crude and adjusted odds ratios (ORs) for the outcomes were calculated using as reference level the occurrence of the outcome among children who never attended an indoor or outdoor chlorinated pool before the age of 2 yrs. A backward approach was used, including all potential control variables and removing the least significant predictor until the model only contained variables with a p-value of <0.20. A total of 24 potential predictors were tested, including, among others, age, sex, parental asthma and/or respiratory allergies (hay fever or allergic rhinitis), high parental educational level (father and/or mother graduate of university or high school), maternal smoking during pregnancy, parental smoking at home, season

of birth, birthweight, number of older siblings, house cleaning with bleach, breastfeeding, day-care centre attendance, area of current residence (Liège *versus* Brussels area), having spent infancy in a urban or rural area, living in the vicinity of a polluting industry or within a distance of 100 m of a busy road, and cumulative time spent in indoor or outdoor chlorinated swimming pools before the age of 2 yrs, separately or in combination (CPA). A classification of CPA in tertiles was also used. For outcomes other than bronchiolitis, CPA at outdoor or indoor swimming pools after the age of 2 yrs was added to the potential predictors. Statistical analyses were performed using SAS version 9.1.3 (SAS, Cary, NC, USA).

RESULTS

Table 1 compares the characteristics of infant swimmers in indoor or outdoor chlorinated pools (n=195) and children who never swam before the age of 2 yrs (n=235). On average, infant swimmers had spent 6 h in indoor and 8 h in outdoor chlorinated pools before the age of 2 yrs. These infant swimmers also showed a greater lifetime CPA at indoor or outdoor chlorinated swimming pools (total indoor and outdoor CPA 132 *versus* 35 h in other children) (table 1). There were no significant differences between the two groups regarding most risk factors of respiratory disease, including sex, age, body mass index, birthweight, exposure to tobacco smoke and parental allergies. The social class or socioeconomic status, as evaluated on the basis of parental education, and the proportions of children with parental asthma, who had been breastfed or attended a day-care centre were higher in the group of swimmers than in non-swimmers. The two groups did not differ with respect to the prevalences of wheezing and ever-diagnosed asthma, respiratory allergies, bronchitis, sinusitis and pneumonia. The prevalence of bronchiolitis was, however, significantly greater in infant swimmers than in their controls (36.4 *versus* 23.8%; p=0.004).

The logistic regression analysis confirmed the association between infant swimming and the risk of bronchiolitis (OR 1.68; 95% CI 1.08–2.68; p=0.03). Other risk factors of bronchiolitis identified included male sex (OR 1.65; 95% CI 1.05–2.59; p=0.03), parental asthma and/or respiratory allergies (OR 1.73; 95% CI 1.20–2.74; p=0.02), number of older siblings (OR 1.69; 95% CI 1.07–2.67; p=0.02), maternal smoking during pregnancy (OR 1.80; 95% CI 1.01–3.37; p=0.05) and day-care centre attendance (OR 1.87; 95% CI 1.17–3.00; p=0.009). Breastfeeding, by contrast, was associated with a protective effect towards bronchiolitis (OR 0.58; 95% CI 0.34–0.97; p=0.04). The parental educational level, a surrogate of socioeconomic status, the use of bleach for house cleaning or parental smoking at home did not emerge as significant predictors of bronchiolitis (p>0.20 for all).

The analysis was pursued by examining whether or not the risk of bronchiolitis was different when attending indoor or outdoor chlorinated pools. As shown in table 2, the OR for bronchiolitis increased dose-dependently with CPA at both types of swimming pool considered separately (p-value for trend <0.05 for both). Stratification of infant swimmers into tertiles of CPA at indoor or outdoor chlorinated pools led to similar patterns of significant increases (p-value for trend 0.009 for indoor and 0.03 for outdoor pools; data not shown). The same pattern of exposure-related increases was found when the analysis was based on children attending only indoor or only outdoor pools to the exclusion of the other type of pool.

TABLE 1 Characteristics of children who swam during infancy and their controls

	Swimming infants	Controls	p-value
Subjects n	195	235	
Males	105 (53.9)	124 (52.8)	0.82
School participation rate %	54.9±19.4	58. 3±23.0	0.19
Age yrs	5.60±0.38	5.68±0.38	0.79
BMI kg·m^{-2}	20.9±3.8	20.6±3.5	0.39
Parents			
Higher educational level	154 (79.0)	156 (66.4)	0.004
Asthma	38 (19.5)	25 (10.6)	0.01
Asthma and/or respiratory allergies	86 (44.1)	88 (37.4)	0.16
Early life			
Birthweight g	3206±639	3436±2396	0.30
Breastfeeding	161 (82.6)	169 (71.9)	0.01
Day-care centre attendance	127 (65.1)	102 (43.4)	<0.001
Exposure to tobacco smoke			
During pregnancy	31 (16.0)	32 (13.6)	0.51
Parental smoking at home	55 (28.2)	74 (31.5)	0.46
CPA before age of 2 yrs h			
Indoor	6 (0–21)		
Outdoor	8 (0–31)		
Total	22 (8–50)		
CPA over lifetime h			
Indoor	53 (17–129)	18 (0–48)	<0.001
Outdoor	50 (4–146)	0 (0–28)	<0.001
Total	132 (52–202)	35 (5–91)	<0.001
Environment and lifestyle			
Number of older siblings	0.87±0.79	0.92±1.12	0.66
House cleaning with bleach	43 (22.1)	66 (28.1)	0.15
Living <100 m from a busy road	63 (32.3)	79 (33.6)	0.78
Exposure to pets since birth	85 (43.6)	99 (42.1)	0.76
Aeroallergen-specific nasal IgE[#]			
House dust mite	21 (12.2)	22 (11.0)	0.71
Cat	15 (8.7)	14 (7.0)	0.54
Pollen	27 (15.7)	27 (13.5)	0.55
At least one aeroallergen	42 (24.4)	48 (24.0)	0.92
Respiratory symptoms and diseases[¶]			
Wheezing[+]	36 (18.5)	40 (17.0)	0.70
Asthma	16 (8.2)	15 (6.4)	0.47
Hay fever	17 (8.7)	27 (11.5)	0.35
Allergic rhinitis	27 (13.9)	25 (10.6)	0.31
Bronchiolitis	71 (36.4)	56 (23.8)	0.004
Bronchitis	93 (47.7)	111 (47.2)	0.92
Sinusitis	28 (14.4)	30 (12.8)	0.63
Pneumonia	20 (10.3)	28 (11.9)	0.59

Data are presented as mean±SD, n (%) or median (interquartile range) unless otherwise indicated. BMI: body mass index; CPA: cumulative pool attendance; Ig: immunoglobulin. [#]: 172 children were tested in the swimming infant group and 200 in the other group; [¶]: defined as doctor-diagnosed diseases at any time; [+]: episodes of wheezing during the last 12 months.

TABLE 2 Risk of bronchiolitis according to cumulative pool attendance (CPA) at indoor or outdoor chlorinated swimming pools during infancy[#]

CPA category	CPA h	Subjects n/N (%)	OR (95% CI)		p-value	p_{trend}
			Unadjusted	Adjusted		
Indoor pool total[¶]						
0 h	0	76/287 (26.5)	1.0 (1.0–1.0)	1.0 (1.0–1.0)		
>0–20 h	6 (2–12)	27/92 (29.4)	1.15 (0.69–1.94)	0.79 (0.44–1.42)	0.44	
>20 h	32 (25–45)	24/51 (47.1)	2.47 (1.34–4.54)	2.02 (1.01–4.02)	0.05	0.006
Outdoor pool total[¶]						
0 h	0	79/308 (25.7)	1.0 (1.0–1.0)	1.0 (1.0–1.0)		
>0–20 h	9 (5–14)	21/54 (38.9)	1.85 (1.01–3.38)	1.91 (0.99–3.68)	0.05	
>20 h	47 (30–84)	27/68 (39.7)	1.91 (1.1–3.3)	1.59 (0.85–2.98)	0.14	0.006
Indoor pool alone						
0 h	0	53/235 (22.6)	1.0 (1.0–1.0)	1.0 (1.0–1.0)		
>0–20 h	6 (2–11)	11/50 (22.0)	0.9 (0.43–1.88)	0.58 (0.25–1.32)	0.19	
>20 h	36 (24–66)	12/23 (52.2)	3.49 (1.46–8.34)	3.49 (1.30–9.34)	0.01	0.01
Outdoor pool alone						
0 h	0	59/235 (25.1)	1.0 (1.0–1.0)	1.0 (1.0–1.0)		
>0–20 h	9 (4–12)	9/27 (33.3)	1.6 (0.68–3.76)	1.69 (0.66–4.3)	0.27	
>20 h	50 (39–76)	11/25 (44.0)	2.51 (1.08–5.85	2.08 (0.81–5.34)	0.13	0.03

CPA is presented as median (interquartile range). Odds ratios (ORs) were adjusted for sex, breastfeeding, day-care centre attendance, number of older siblings, parental asthma and/or respiratory allergies, maternal smoking during pregnancy, and, when the analysis was performed on all children, attendance of the other type of swimming. CI: confidence interval; N: number of subjects in group; p_{trend}: p-value for trend. [#]: before the age of 2 yrs; [¶]: all children.

It was also ascertained that these associations were not confounded by differences in participation rate between schools or by other risk factors for bronchiolitis, especially those linked to the socioeconomic status of the children, which, indeed, was higher in infant swimmers than in controls. This analysis was conducted by combining the CPA at indoor and outdoor pools in order to obtain sufficient subjects in the different pool attendance categories. As shown in table 3, associations between bronchiolitis and infant swimming persisted, and were very consistent (OR for CPA of >20 h was 2.2–2.7, $p \leqslant 0.03$) across categories created by excluding children from schools with a low participation rate (<50%), children from parents with a lower educational level, children who had not been breastfed, children who had been exposed to tobacco smoke or else who had lived with older siblings or in a house cleaned with bleach. Quite interestingly, exclusion of children of parents with asthma or respiratory allergies, or of children having attended a day-care centre, two well-known risk factors for bronchiolitis, noticeably strengthened the associations between bronchiolitis and infant swimming (OR for CPA of >20 h 4.45 and 4.44; p=0.001 and 0.007, respectively), which emerged through a particularly remarkable dose–response relationship among children who had never attended a day-care centre (p<0.001).

Lastly, the temporal coherence of these associations was checked by comparing the age of bronchiolitis occurrence with the age at which the child started to attend swimming pools. Of the 71 infant swimmers who had bronchiolitis, there were 54 cases for which the parents provided the exact dates when their child started swimming and developed bronchiolitis. On average, these infant swimmers developed bronchiolitis at the age of 9.6±5.9 months and were diagnosed with asthma at the age of 21.8±18.6 months. Among these, bronchiolitis occurred after the start of infant swimming in 35 (65%) cases, a number consistent with the excess of bronchiolitis cases observed in the infant swimming group. Children who never swam during infancy had bronchiolitis at about the same age as infant swimmers (9.3±5.5 months), but were diagnosed with asthma later, at the age 30.6±10.0 months.

Since bronchiolitis is known to increase the risk of wheezing, asthma or allergic sensitisation in subsequent years, the late consequences of bronchiolitis were also compared between infant swimmers and nonswimmers. This comparison was made by adjusting the ORs for CPA at indoor or outdoor pools after the age of 2 yrs, which, indeed, was very different between the two groups. Table 4 clearly shows that bronchiolitis was associated with an increased risk of wheezing, doctor-diagnosed asthma and hay fever only among children who had attended chlorinated pools during their infancy. Interestingly, bronchiolitis and infant swimming also interacted to increase the risk of sensitisation to house dust mite (p=0.04) and pollen (p=0.05). There were no differences in the ORs for cat allergy and allergic rhinitis between the two groups. When considering children who never developed bronchiolitis, there were no significant differences in the prevalences of respiratory diseases and sensitisation to aeroallergens between children who swam during infancy and those who did not (p>0.07 for all; Chi-squared test) (table 4).

DISCUSSION

The present study shows that swimming in indoor and outdoor pools during infancy is associated with an exposure-related increase in the risk of bronchiolitis. This effect is independent of other known risk factors for bronchiolitis, such

TABLE 3 Risk of bronchiolitis with increasing cumulative pool attendance (CPA) at indoor and/or outdoor chlorinated pools during infancy[#] by risk-factor category

	0-h CPA		>0–20-h CPA			>20-h CPA			p_{trend}
	Subjects n/N (%)	OR (95% CI)	Subjects n/N (%)	OR (95% CI)	p-value	Subjects n/N (%)	OR (95% CI)	p-value	
Total	56/235 (23.8)	1.0 (1.0–1.0)	29/95(30.5)	1.24 (0.69–2.23)	0.46	42/100 (42.0)	2.25 (1.32–3.85)	0.003	<0.001
Schools with >50% response rate	35/146 (24.0)	1.0 (1.0–1.0)	17/59(28.8)	1.24 (0.57–2.7)	0.59	23/58 (39.7)	2.25 (1.10–4.59)	0.03	0.03
High parental educational level	36/156 (23.1)	1.0 (1.0–1.0)	22/72(30.6)	1.31 (0.67–2.57)	0.43	34/82 (41.5)	2.26 (1.22–4.18)	0.009	0.003
Parents with no asthma/respiratory allergies	17/115 (14.8)	1.0 (1.0–1.0)	13/38 (34.2)	4.31 (1.55–12.0)	0.005	18/45 (40.0)	4.45 (1.82–10.9)	0.001	0.001
No breastfeeding	19/66 (28.8)	1.0 (1.0–1.0)	7/12 (58.3)	1.06 (0.55–2.06)	0.86	12/22 (54.6)	2.10 (1.11–3.98)	0.02	0.008
No day-care centre attendance	21/133 (15.8)	1.0 (1.0–1.0)	9/32 (28.1)	2.35 (0.87–6.38)	0.09	15/36 (41.7)	4.44 (1.88–10.5)	0.007	<0.001
No maternal smoking during pregnancy	46/203 (22.7)	1.0 (1.0–1.0)	21/77 (27.3)	1.27 (0.65–2.49)	0.48	34/87 (39.1)	2.16 (1.20–3.90)	0.01	0.005
No exposure to parental smoking	38/161 (23.6)	1.0 (1.0–1.0)	19/65 (29.2)	1.16 (0.57–2.35)	0.61	32/75 (42.7)	2.21 (1.18–4.17)	0.01	0.004
No older siblings	16/99 (16.2)	1.0 (1.0–1.0)	13/45 (28.9)	2.26 (0.87–5.88)	0.09	16/47 (34.0)	2.72 (1.13–6.56)	0.03	0.01
No use of bleach for house cleaning	48/201 (23.9)	1.0 (1.0–1.0)	28/87 (32.2)	1.37 (0.75–2.51)	0.30	36/87 (41.4)	2.22 (1.25–3.95)	0.007	0.003

Odds ratios (ORs) were adjusted for breastfeeding, sex, number of older siblings, area of residence, day-care centre attendance, maternal smoking during pregnancy and parental antecedents of atopic disease (except when the factor was excluded). A high level of education means that the father and/or mother graduated from a university or high school. N: number of subjects in group; CI: confidence interval; p_{trend}: p-value for trend.
[#]: before the age of 2 yrs.

as day-care attendance, exposure to tobacco smoke or parental antecedents of atopic diseases. The present study also shows that, among children who had bronchiolitis, only those who were infant swimmers were at greater risk of asthma and respiratory allergies in subsequent years.

These results are in concordance with the observation of NYSTAD and co-workers [9, 10] that swimming before the age of 2 yrs increases the prevalence of lower respiratory tract infection. They are also consistent with a prior study on schoolchildren [11, 12], suggesting that infant swimming may cause airway changes predisposing to asthma and recurrent bronchitis. In the present study focusing on younger children, no significant differences were found between infant swimmers and other children regarding the risk of asthma and allergies. This lack of association between infant swimming and asthma, reported by other authors for children of the same age [18–20], does not necessarily argue against the hypothesis of a causal link between infant swimming and poorer respiratory health later during childhood. Children aged 5–6 yrs are probably too young to detect associations with chronic respiratory diseases that develop and are correctly diagnosed later during childhood. This might explain why studies linking swimming pool attendance to childhood asthma were all based on children with a mean age of >9 yrs [21–23]. The follow-up of the present cohort of infant swimmers should provide more conclusive data regarding the long-term consequences of infant swimming.

The swimming pool factor responsible for the risk of bronchiolitis is hard to identify with certainty given the multiplicity of potentially harmful agents in the swimming pool environment [20, 24]. Currently, one of the most plausible explanations is that the airways of infant swimmers are made more sensitive to infections because of the irritating effects of the chlorine used to disinfect swimming pools. The fact that bronchiolitis is increased by outdoor, as well as by indoor, pools means that chloramines are not the sole and probably not the main irritants that might be implicated in the risk of bronchiolitis. The first reason for this is that the outdoor pools attended by infant swimmers in the present study were mainly residential pools. Compared to public pools, residential pools are, indeed, much less polluted by organic matter from bathers, in particular by urine, which is the main source of nitrogen leading to the formation of chloramines. The second reason is that trichloramine, the ultimate chlorination by-product, is a highly volatile gas that is very quickly dispersed into the atmosphere once released at the surface of open-air pools, which, therefore, do not have the characteristic chlorine smell of indoor pools. As for the risks of asthma and respiratory allergies associated with outdoor pools [15], we suspect that the major burden of oxidants irritating the airways of infant swimmers comes from the microaerosols or small volumes of water that they inhale while actively playing and having their head under water [12]. The risk of inhaling small volumes during submersion exercise is especially important since infants cannot control their breathing. If the infant swimming practice is considered to be safe, it is because of the laryngeal or gag reflex (closure of the larynx with the epiglottis) that is triggered when water gets into the infant's mouth. This gag

TABLE 4 Risk of respiratory diseases and sensitisation to aeroallergens associated with bronchiolitis in swimming infants and their controls

	Controls					Swimming infants				
	Bronchiolitis n (%)		OR (95% CI)		p-value	Bronchiolitis n (%)		OR (95% CI)		p-value
	No	Yes	Unadjusted	Adjusted		No	Yes	Unadjusted	Adjusted	
Subjects n	179	56				124	71			
Wheezing	28 (15.6)	12 (21.4)	1.47 (0.69–3.13)	1.29 (0.47–3.54)	0.62	18 (14.5)	18 (25.4)	2.00 (0.96–4.16)	2.49 (1.13–5.49)	0.03
Asthma	11 (6.2)	4 (7.1)	1.18 (0.36–3.85)	0.91 (0.23–3.70)	0.90	5 (4.0)	11 (15.5)	4.36 (1.45–13.1)	8.27 (2.30–29.6)	0.001
Allergic rhinitis	16 (8.9)	9 (16.1)	1.95 (0.81–4.70)	2.08 (0.79–5.40)	0.14	13 (10.5)	14 (19.7.)	2.10 (0.92–4.76)	4.65 (1.42–15.2)	0.01
Hay fever	19 (10.6)	12 (21.4)	1.40 (0.58–3.41)	0.96 (0.34–2.70)	0.94	6 (4.8)	11 (15.5)	3.61 (1.27–10.2)	4.70 (1.52–14.5)	0.007
Nasal IgE										
Pollen[#]	24 (15.7)	3 (6.4)	0.39 (0.11–1.38)	0.57 (0.15–2.10)	0.40	14 (12.8)	13 (20.6)	1.86 (0.80–4.30)	2.63 (1.01–6.80)	0.05
HDM[#]	15 (9.8)	7 (14.9)	1.62 (0.62–4.25)	2.05 (0.62–6.75)	0.24	9 (8.3)	21 (33.3)	2.59 (1.02–6.55)	2.86 (1.07–7.80)	0.04
Cat[#]	11 (7.2)	3 (6.4)	0.89 (0.24–3.32)	0.90 (0.20–4.18)	0.90	8 (7.3)	7 (11.1)	1.56 (0.54–5.54)	1.26 (0.40–3.96)	0.69

Outcomes are defined in table 1. Bronchiolitis was diagnosed at a mean±SD age of 9.6±5.9 months in infant swimmers and 9.3±5.5 months in controls. The prevalences of respiratory diseases and sensitisation to aeroallergens among children who never developed bronchiolitis did not differ significantly between swimming infants and their controls ($p>0.07$ for all; Chi-squared test). OR: odds ratio; CI: confidence interval; Ig: immunoglobulin; HDM: house dust mite. [#]: a total of 153 and 47 controls and 109 and 63 swimming infants, respectively, who did not or did develop bronchiolitis, were tested.

reflex disappears, however, when the infant gets older (>6 months), and, anyway, this reflex cannot prevent small amounts of chlorinated water deposited or trapped in the upper respiratory tract being conducted more deeply into the lungs when the infant surfaces to breathe [12].

The most reactive and concentrated chlorine compound present in the water and microaerosols of both outdoor and indoor pools is hypochlorite/hypochlorous acid, *i.e.* the active chlorine itself (concentrations ranging 1–2 ppm). Depending on the level of organic pollution of pool water, infant swimmers have also been exposed to chlorination by-products, among which the most irritating and concentrated are mono-, dichlor- and trichloramine, as well as dichloromethylamine [19, 20, 24, 25]. Since public pools are usually more polluted by nitrogenous substances from bathers (urine, sweat and saliva) than residential pools, irritating effects caused by the chloramines might explain why the ORs for bronchiolitis are higher with indoor than with outdoor pools.

Chlorine-based oxidants, such as hypochlorous acid or chloramines, are known to be potent oxidants capable of damaging the endothelial and epithelial barriers [26, 27]. Studies based on lung injury markers have shown that, at concentrations commonly found in indoor pools, these chemicals can affect the permeability or the cellular integrity of the deep lung epithelium. Regular attendance at chlorinated pools by schoolchildren has been associated with an exposure-dependent increase in lung epithelium permeability (lung hyperpermeability), resulting in intravascular leakage of surfactant-associated proteins A and B [28]. A decrease in serum CC16 has also been described in children who use indoor pools during their infancy or later in childhood, which is a reflection of a decrease in Clara cell numbers and an ensuing decrease in CC16 production [11, 12, 14]. The latter observation is particularly relevant to the present study given the experimental evidence that CC16 downregulates inflammation during acute RSV infection [13].

Even though airways irritation by chlorine-based oxidants appears to be a very likely explanation for the increased risk of bronchiolitis associated with infant swimming, other causative factors might also play a role. As a result of inadequate disinfection with chlorine, the swimming pool environment can be contaminated by viruses (adeno-, noro- and echovirus), which may cause outbreaks of water-borne illnesses, such as gastroenteritis, dermatitis and respiratory infections [29]. Respiratory illnesses in these incidents are rather uncommon, and, in most cases, limited to the upper airways. Nevertheless, we have no evidence permitting us to exclude the possibility that the swimming pool environment may increase the risks of cross-infection with RSV [30, 31]. Another possibility that should be considered is the inhalation of swimming pool water when infants perform submersion activities. Owing to its hypotonicity, swimming pool water deposited in airways might, perhaps, cause some airway alterations, aggravating the toxic effects of chlorination products [32].

The present study has certain limitations. The most important one concerns exposure assessment, since it was obviously not possible to retrieve exposure data regarding the levels of chlorine in the indoor or outdoor swimming pools that the children attended during infancy. There was no choice but to use the information provided by the parents. The existence of exposure–response relationships for both outdoor and indoor pools suggests that the lack of data regarding the precise levels of chlorine in swimming pools has not been critical to the point of distorting the present analysis. Another limitation concerns the participation rate, which only reached 51% overall and varied greatly between schools. The young age of the children

combined with the fact that the medical examination, though based on noninvasive tests, was performed in schools probably deterred a significant proportion of parents from participating in the study. This low participation rate does not seem to have biased the study since there were no significant correlations between participation rate and the prevalences of the main outcomes and risk factors. The response rate, tested along with other potential confounders, did not emerge as a significant predictor of bronchiolitis either. This lack of confounding by the participation rate probably stems from the fact that parents were blinded to the tested hypothesis. Since the study was conducted in the framework of a prospective study on environmental factors affecting the respiratory health of children, infant swimming was only one of the many items addressed by the questionnaire.

In summary, the present study shows that infant swimming is associated with a dose-dependent increase in the risk of bronchiolitis. Exposure to chlorinated pools during infancy also interacts with bronchiolitis to increase the risks of asthma and respiratory allergies later during childhood, which suggests that the infant swimming practice may have a more long-standing impact on the respiratory health of children.

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STATEMENT OF INTEREST

None declared.

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available reports. The findings in the ALSPAC cohort, the first prospective study focusing on the asthma risk related to chlorinated swimming pools, are reassuring, however, and suggest that recreational swimming in chlorinated pools does not raise the risk of asthma and allergic symptoms in children. The relevance of this topic to public health, nonetheless, means that further longitudinal studies are certainly warranted to reinforce the evidence in favor of the safety of children swimming in public pools.

At this point, we would encourage the authors of the *Wikipedia* page on the "pool chlorine hypothesis" (1) to reconsider their alarming report, possibly adopting a more positive and reassuring tone for recreational swimmers.

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Con: Respiratory Risks Associated with Chlorinated Swimming Pools

A Complex Pattern of Exposure and Effects

Chlorine used to disinfect swimming pools, and its derivatives, are well-known irritants of the skin, eyes, and of the upper respiratory tract. For a long time, the irritating effects of these substances have been regarded as a mere source of discomfort for swimmers and unlikely to cause adverse effects outside accidental exposures. In this issue of the *Journal* (pp. 582–588), Font-Ribera and coworkers report the results that support this view (1). These authors studied a cohort of 5,738 children born in 1991–1992 (the ALSPAC cohort) who were followed up until the age of 10 years. Data collected by questionnaire until the age of 7 years show no increase of ever-diagnosed asthma with the frequency of swimming, even when considering only children with atopy. In addition, no association was found with atopy screened at age 7–8 years, or with any current asthma or respiratory symptom that were recorded till the age of 10 years. There was even an improvement of lung function measured at 7–8 years, and at 10 years a decreased risk of asthma symptoms was seen in children with pre-existing respiratory conditions.

And yet, the idea that chlorination products (CPs) contaminating the air of swimming pools pose no risk to swimmers is increasingly challenged by reports of respiratory problems among swimming pool attendees. Studies conducted in France (2), in The Netherlands (3), and in Italy (4) clearly show that swimming pool workers, and in particular lifeguards and trainers, are at increased risk of developing irritation and asthma symptoms. The agent responsible for the effects is most probably trichloramine (also called nitrogen trichloride), the gas that gives indoor pools their typical smell and can induce occupational asthma in lifeguards (5). Regarding swimmers, it has been known for more than two decades that elite swimmers present a higher prevalence of asthma, rhinitis, and respiratory allergies than do other athletes (6–8). This poorer respiratory health of

swimmers is partly attributable to the selection bias due to the lower asthmagenicity of indoor swimming compared with other sports (8). However, researchers now increasingly acknowledge that these respiratory problems may be caused by chlorine used to disinfect pool water (9, 10). Concern about the dangers of chlorinated pools was really aroused with the finding in a Belgian study that the attendance at indoor chlorinated pools correlated with lung epithelium hyperpermeability and asthma prevalence in schoolchildren (11). Further studies in Belgium showed that the asthma risk linked to swimming pools stems from an interaction between pool attendance and atopic status, the asthma prevalence increasing dose-dependently only among subjects with elevated serum immunoglobulin E (IgE) or with aeroallergen-specific serum IgE (12, 13). A similar pool chlorine–atopy interaction in asthma risk was found in swimmers attending outdoor chlorinated pools, which suggests that the major burden of irritants inhaled by swimmers comes from the gaseous or aerosolized CPs building up at the surface of all chlorinated pools (14). These respiratory effects and similar observations made by other researchers (15–18) support the chlorine hypothesis, which postulates that the rise of asthma and allergic diseases in developed countries could be driven to a large extent by the increasing exposure of children to CPs from swimming pools. Consistent with this chlorine hypothesis, an ecological study across Europe has brought to light strong associations between childhood asthma prevalence and the availability of indoor chlorinated swimming pools (19).

The pattern of respiratory effects associated with chlorinated pools might be much broader and also more complex than suggested by these first studies that were focused on asthma. It increasingly appears that chlorinated pools might also promote the development of common upper airways allergic diseases such as hay fever and allergic rhinitis. In a retrospective analysis of a cohort of adults, Kohlhammer and colleagues (20) found that early school pool attendance was associated with an increased risk of hay fever. In our recent study among adolescents, we also found that the prevalence of doctor-diagnosed hay fever increased dose-dependently with the time spent in chlorinated pools. The possibility even emerges that when exposure is very early, chlorinated pools might exert an adjuvant effect in the sensitization process itself (i.e., on the development of atopy). An increased risk of sensitization to aeroallergens, and especially to house dust mite, was observed in adolescents having regularly available outdoor chlorinated pools before the age of 7 years (14), and a similar finding was made in children having ever attended an outdoor pool before the age of 2 years (15). Further complicating the picture, studies in Norway (21, 22) and Belgium (23, 24) provide quite consistent data suggesting that attendance at chlorinated pools during infancy increases the risk of lower respiratory tract infection and especially of bronchiolitis. In a recent Belgian study (24), infant swimmers who had bronchiolitis had a much greater risk of developing asthma and respiratory allergies in subsequent years than infants who never swam during infancy. The case for implicating chlorine in these increased risks of respiratory diseases is strengthened by observations among subjects having attended a swimming pool disinfected by the copper-silver ionization method. The prevalences of asthma and respiratory allergies in these subjects did not correlate with pool attendance and were about two to three times lower than those of their peers who swam regularly in chlorinated pools (13). The current hypothesis, supported by biomarker studies on swimmers (11, 16, 23, 25, 26), is that CPs inhaled in the form of gases, aerosols, or even water can cause structural and functional changes in airways epithelium that, alone or interaction with other risk factors, predispose swimmers to respiratory allergies and infections.

From a mechanistic point of view, epidemiologic studies have brought to light two important sources of variation in exposure–response relationships, which make associations between swimming and respiratory outcomes particularly difficult to detect without a precise exposure assessment. First, the shape and the dose scale of exposure–response relationships vary with the type of respiratory outcome. For instance, the interaction between chlorinated pool attendance and atopy in the risk of hay fever appears to be triggered by much lower cumulative pool attendance than the risk of asthma (13). This difference is probably linked to the variable deposition of CPs along the respiratory tract, and thus to the fact that the upper airways receive the major burden of irritants inhaled by swimmers. Second, exposure–response relationships appear to vary noticeably also with the timing of exposure (i.e., the age of swimmers). The risk of childhood asthma associated with chlorinated pool attendance increases as pool attendance is accumulated over increasingly lower ages, which in other terms means that the exposure–response relationships are shifted to increasingly lower exposures as the swimmer's age decreases (12). In infants and very young children, the risks of respiratory infection and allergic sensitization were even found to increase after only a few tens of hours spent in chlorinated pools (15, 23).

If, as suggested by the above studies, CPs in swimming pools can be detrimental to the airways of swimmers, how can one explain that the study by Font-Ribera and coworkers (1) provides, if anything, evidence suggesting a protective effect of swimming? We see two possible explanations. The first explanation is linked to the timing of outcomes development. Because of the differences in exposure–response relationships outlined above and also the natural course of respiratory diseases, respiratory outcomes associated with chlorinated pool attendance require different periods of time to be realized. If risks of respiratory infections or allergic sensitization can be triggered during infancy or childhood after relatively low attendance at chlorinated pools, the risk of asthma has been described so far only in atopic children more than 10 years of age. No direct link between asthma and swimming has been reported for younger children like those examined in the study by Font-Ribera and colleagues (24, 28). Children of that age are probably too young to detect associations with a chronic disease like atopic asthma, which develops and is usually correctly diagnosed later during childhood. The second possible explanation for the negative findings in the ALSPAC study is an underestimation of exposure, with a corollary of misclassification of some categories of swimmers. It is indeed important to realize that data of the ALSPAC cohort were collected in the 1990s and thus before the first report in 2003 (11) suggesting a link between swimming and childhood asthma. Clearly, the ALSPAC questionnaire was not designed for an accurate assessment of the time spent in indoor or outdoor chlorinated pools. When one compares the climate and socioeconomic status of the United Kingdom and Belgium, there are no reasons to believe that Belgian children should attend swimming pools much more frequently than their British peers. If a difference in swimming pool attendance did exist between these two countries, it would be actually the opposite trend, given the popularity of swimming in the United Kingdom. And yet, only 15% of the ALSPAC children had ever attended a swimming pool at the age of 4 years, while in our recent study 45% of schoolchildren had ever attended a chlorinated swimming pool by the age of 2 years (24). The pool attendance of the ALSPAC children between the age of 4 and 7 years has probably also been underestimated, since it was mainly based on the attendance of children in school, which in our

different cohorts represents less than 50% of the total swimming pool attendance.

In conclusion, given the complexity of the exposure and effect pattern when attending chlorinated pools, future studies should be based on very accurate estimates of the cumulative exposure to CPs regardless of the type of pool and frequency of attendance. These studies should also focus on the consequences of exposure during infancy and childhood, when developing airways are most vulnerable to toxic injury. More research is also needed to explore the mechanisms by which changes in airways epithelium observed in swimmers might predispose to the development of respiratory diseases. In the meantime, we strongly recommend the implementation of measures to minimize exposure to CPs in swimming pools. We think that it is very unlikely that the excess of respiratory ailments observed in swimmers and other pool attendees could arise merely by bias or chance.

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Biomarkers of early respiratory effects in smoking adolescents

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ABSTRACT: Noninvasive biomarkers can be used to evaluate airways damage caused by tobacco smoke, but studies so far have only involved adult smokers. In this study, we evaluated whether such biomarkers can detect early respiratory effects in adolescents passively or actively exposed to tobacco smoke.

In a cross-sectional study of 845 adolescents (mean age 16 yrs), we measured exhaled nitric oxide (NO) and various epithelial markers in nasal lavage fluid (NALF) and serum, including Clara cell protein (CC16) and surfactant protein (SP)-D. Information about smoking habits and potential confounders was collected by questionnaire. Four groups of equal size (n=36), of nonsmokers, passive smokers, light smokers (<5 cigarettes·day^{-1}, median 0.08 pack-yrs) and heavy smokers (⩾5 cigarettes·day^{-1}, median 0.35 pack-yrs), were matched using an automated procedure.

The levels of exhaled NO and of CC16 in NALF were significantly decreased in the group of heavy smokers. A trend towards lower levels of CC16 in NALF was observed in passive smokers. There were no significant changes in serum CC16 and SP-D, which suggests that the deep lung epithelium had not yet been affected by smoking.

In conclusion, tobacco smoke can cause early changes in the airways of adolescents with a cumulative smoking history of <1 pack-yr.

KEYWORDS: Adolescents, biomarkers, cigarette smoke, Clara cell protein 16, nasal lavage fluid, nitric oxide

Over recent years, several approaches have become available to assess the response of the respiratory system to inhaled agents. Bio-imaging techniques and functional assays have undergone significant improvements, but progress has also been achieved using noninvasive biomarker methods, *e.g.* the analysis of exhaled breath, the detection of inflammatory cells and mediators in induced sputum, and the analysis of lung-specific proteins in serum [1–4]. Recently, analysis of biomarkers collected after nasal lavage has also received increased attention [5]. Multiple studies have been published showing changes in biomarker levels in diseased individuals, *e.g.* increased exhaled nitric oxide (NO) during asthma exacerbations, increased levels of surfactant protein (SP)-D in serum of chronic obstructive pulmonary disease (COPD) patients or decreased levels of Clara cell protein (CC16) in serum or induced sputum of COPD patients [6–8].

One of the most valuable applications of biomarkers is the detection of preclinical phases of disease development. In this respect, asymptomatic smokers are particularly interesting, as they represent a large group of subjects chronically exposed to agents having clear detrimental effects on human health [9, 10]. Structural and functional changes in the nose have been described in smokers, starting from 10 pack-yrs [11]. Smokers show decreased levels of serum CC16 [12], which can already be detected in young adults with a median of only 3.8 pack-yrs [13]. The reduced levels of serum CC16 in smokers most probably reflect a decrease of CC16 in bronchoalveolar lavage fluid paralleling the progressive loss of CC16-positive cells in terminal airways [14]. The concentrations of SP-A and -B, conversely, rise in the serum of smokers [12] as a consequence of the disruption of epithelial barriers by tobacco smoke. Smokers also have a decreased concentration of NO in the exhaled breath (mean -52%, average 10.2 pack-yrs), which correlates with their daily cigarette consumption ($r=0.77$, $p<0.001$) [15]. Decreased levels of exhaled NO fraction were also observed in infants exposed pre- and post-natally to cigarette smoke compared with never-exposed infants or infants exposed to tobacco smoke only after birth [16]. In agreement with these biomarker studies, LEDERER *et al.* [17] recently reported subclinical parenchymal lung disease using computed tomography and spirometric measurements in a generally healthy cohort of older adult smokers.

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Until now, biomarker studies among smokers have been conducted on adult subjects with a smoking history of at least several pack-years. Here, we investigated whether noninvasive biomarkers of the upper and lower respiratory tract can detect subclinical changes in the airways of adolescents with an active or passive exposure to cigarette smoke.

MATERIAL AND METHODS

Study population

The study protocol was approved by the Ethics Committee of the Faculty of Medicine of the Catholic University of Louvain (Brussels, Belgium) and complied with all applicable requirements of international regulations. Details about the cross-sectional study on adolescents in Belgium have been described elsewhere [18]. Briefly, the study was conducted among adolescents in the third and fourth grades of secondary school. A questionnaire and a written agreement to participate in the study were obtained from the adolescents' parents. The questionnaire addressed aspects related to social and medical characteristics of the adolescent and their family, and to the in-house and out-of-house environment.

Examination of study participants

The adolescents participating in the study were examined in their school. Examination took place between ~09:00 and 15:00 h. For each test or sample collection, the time was recorded to adjust for possible diurnal variations in the biomarker levels. The examination consisted of an interview enquiring about the smoking status and recent (<12 months') respiratory symptoms, followed by the measurement of height and weight, exhaled NO levels and lung function parameters, and the collection of a blood and of two nasal lavage fluid (NALF) samples. The smoking status used for grouping the study participants was based on the information provided by the adolescents themselves. The active smokers were divided into ''light smokers'' and ''heavy smokers'' depending on the daily cigarette consumption (<5 or $\geqslant$5 cigarettes, respectively). The allocation criterion of 5 cigarettes·day^{-1} was selected for pragmatic reasons, *i.e.* it allowed definition of a group of heavy smokers of sufficient size and a slightly larger group of light smokers, better enabling the matching procedure (see statistical analyses section). A sample of venous blood was collected in a dry tube, allowed to clot overnight at 4°C and then centrifuged at 2,000 × g for 10 min. Serum was decanted and stored at -18°C until biomarker analysis. NALF samples were collected from both nostrils. Participants were asked to sit down, bend forward and put their heads down. 2.5 mL of sterile physiological saline at 37°C were instilled into each nostril by a disposable tip connected to a peristaltic pump. After 10 s, students were asked to lift their head and the lavage fluid was collected using a small funnel. The NALF samples were stored at -20°C until evaluation. The concentration of NO in exhaled air was determined with the NIOX^{TM} analyser (Aerocrine AB, Solna, Sweden) according to the guidelines of the American Thoracic Society [19]. Total and aeroallergen-specific immunoglobulin (Ig)E (house-dust mite, cat epithelium, dog dander, moulds, tree pollen, grass pollen and herbaceous pollen mixture) concentrations in serum were determined using the Immulite® IgE kit (Diagnostic Products Company, Los Angeles, CA, USA). Sensitisation against specific aeroallergens was defined as a serum concentration of specific IgE >0.35 kIU·L^{-1}. CC16 in serum and NALF was measured by latex immunoassay using a rabbit anti-CC16 antibody (Dakopatts, Glostrup, Denmark) and standard CC16 purified in our laboratory at the Louvain Centre for Toxicology and Applied Pharmacology, Faculty of Medicine, Catholic University of Louvain [20]. The serum concentration of SP-D was determined using a commercially available ELISA kit (code no. YSE-7744; Yamasa Corporation, Choshi, Japan). Albumin, creatinine and urea were quantified by the Beckman Synchron CX5 Delta Clinical System (Beckman Coulter Inc., Fullerton, CA, USA). Concentrations of biomarkers in NALF were adjusted for the variable dilution of the recovered epithelial lining fluid, either by calculating the absolute amount of recovered protein or by adjusting the concentration in NALF with the plasma/NALF concentration ratio of urea [21, 22]. These adjustments were made for each nostril separately and then the mean value was calculated and used for the statistical analyses.

Statistical analyses

Matching procedures and statistical analyses were performed using the R statistical package (The R Project, Institute for Statistics and Mathematics, Vienna University of Economics and Business, Vienna, Austria) [23–26]. The matching was performed relative to the group of heavy smokers, which included 36 subjects, except for biomarkers in NALF for which we excluded the adolescents who had a cold (n=9) during the previous 2 weeks. Groups equivalent to the heavy smokers group were generated from the total groups of nonsmokers (n=507), passive smokers (n=254) and light smokers (n=48) using an automated matching procedure (''optmatch package'') based on the following characteristics: ''sex'', ''age'', ''parental allergy or asthma'' and ''parent with higher education''. Associations of the smoking status with the personal and familial characteristics, medical characteristics or antecedents, the self-reported respiratory symptoms, sensitisation and characteristics of the in-house and out-of-house environment were analysed by pair-wise comparison with the nonsmokers group using the Chi-squared test. Comparisons of the continuous variables with the nonsmokers group were performed using one-way ANOVA followed by Dunnett's *post hoc* test. Models for biomarkers in NALF, blood and exhaled air were built by means of generalised linear modelling using the Gaussian distribution. The initial models were built assessing the association of each biomarker with being a light smoker, a heavy smoker or a passive smoker, with age, sex, body mass index (BMI), birth weight, total serum IgE concentration, time of sample collection, the social and medical characteristics and variables reflecting the in-house and out-of-house environment quality (*e.g.* siblings, mould on bedroom wall, wood smoke, air fresheners, cleaning with bleach). These models were run by also testing the interactions between sex and the different smoking statuses (passive smoker × sex, light smoker × sex, and heavy smoker × sex). For serum markers, we included in the models the serum creatinine concentration to account for the influence of renal function. The obtained models were optimised using a backward stepwise algorithm using the Akaike Information Criterion (AIC; R-function: ''step''). For each of the investigated outcomes, the model with the lowest AIC score was retained. Statistical procedures on continuous variables were performed after log-transformation. Graphs and statistics on adjusted concentrations were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Relationships between biomarkers and

cigarette consumption were assessed using the Spearman nonparametric correlation test. The threshold for statistical significance was set at $p<0.05$.

RESULTS

Characterisation of subpopulations

An overview of the characteristics of the different subpopulations is given in table 1. There were no or very few differences between the groups regarding sex, age, BMI and birth weight. As expected from adolescents who started smoking recently, the cumulative cigarette consumption was very low, with a median of 0.08 and 0.35 pack-yrs for the light and heavy smokers, respectively. Despite the matching procedure, a few differences persisted between the groups, in particular concerning the place of residence and the exposure to older siblings. The different groups had similar prevalences of allergic sensitisation or of ever-diagnosed asthma, hay fever and lower respiratory tract infections. The prevalence of allergic rhinitis and frequent cold tended to be higher among heavy smokers. Compared with the nonsmokers, all smoker groups showed a trend towards higher prevalences of upper respiratory symptoms such as cough crisis, nasal problems and wheezing.

Epithelial biomarkers

Table 2 shows the results of the univariate analyses of biomarkers. The NALF volumes as well as the urea concentrations were not significantly different between the nonsmokers and the other groups. The median CC16 concentration in NALF was decreased in all smoker groups, reaching statistical significance for the heavy smokers group (median 61% lower than nonsmokers). A similar pattern of significant decreases was found after adjustment for the variable dilution of NALF sample by calculating the

TABLE 1 Overview characteristics of subpopulations

	Nonsmokers	Passive smokers	Light smokers#	Heavy smokers¶	p-value
Female	23 (64)	29 (81)	22 (61)	23 (64)	0.58
Age yrs	16.1 (15.9–16.7)	16.2 (15.8–16.8)	16.0 (15.7–16.6)	16.0 (15.8–17)	0.83
Birth weight kg	3.50 (3.28–3.65)	3.32 (3.04–3.63)	3.30 (3.09–3.46)	3.25 (2.96–3.60)	0.87
BMI kg·m^{-2}	20.9 (19.3–23.2)	20.5 (19.1–23.3)	20.6 (19.4–22.3)	20.3 (19.4–22)	0.84
Cigarette consumption					
Cigarettes·day^{-1}	0	0	1.8 (0.3–3.5)	7.3 (5.5–10.0)	ND
Pack-yrs	0	0	0.08 (0.01–0.18)	0.35 (0.28–0.50)	ND
Parent with higher education	13 (36)	12 (33)	19 (53)	17 (47)	0.15
Parent with allergy or asthma	19 (53)	17 (47)	19 (53)	18 (50)	0.94
Having older siblings	17 (47)	21 (58)	27 (75)*	25 (69)	0.02
Day care attendance	12 (33)	11 (31)	11 (31)	13 (36)	0.81
Living in rural area	27 (75)	23 (64)	28 (78)	18 (50)*	0.08
Having a furred pet	27 (75)	30 (83)	31 (86)	33 (92)	0.053
Use of air fresheners at home	10 (28)	16 (44)	10 (28)	15 (42)	0.48
Bleach as cleaning agent	11 (31)	11 (31)	11 (31)	18 (50)	0.10
Heating system with wood	11 (31)	11 (31)	10 (28)	13 (36)	0.71
Pool attendance >500 h	15 (42)	16 (44)	17 (47)	16 (44)	0.76
Serum IgE					
Total kIU·L^{-1}	39.7 (14–192)	30.3 (8.4–72.9)	40.2 (18.3–155.5)	57.3 (21.2–154)	0.59
Aeroallergen-specific IgE	15 (43)	9 (25)	14 (39)	13 (36)	0.87
Respiratory function and diseases					
FEV_1 % pred	100 (92–108)	103 (94–113)	102 (91–112)	102 (94–111)	0.93
Allergic rhinitis	4 (11)	5 (14)	5 (14)	10 (28)	0.07
Asthma	3 (8)	4 (11)	6 (17)	5 (14)	0.37
Bronchitis	19 (53)	14 (39)	20 (56)	15 (42)	0.65
Bronchiolitis	4 (11)	4 (11)	5 (14)	3 (8)	0.81
Colds >4·yr^{-1}	14 (39)	15 (42)	15 (42)	18 (50)	0.34
Hay fever	8 (22)	5 (14)	6 (17)	5 (14)	0.42
Respiratory symptoms					
Chest tightness	3 (8)	1 (3)	6 (17)	3 (8)	0.52
Cough crisis	5 (14)	7 (19)	15 (42)*	14 (39)*	0.004
Nasal problems	11 (31)	20 (56)*	14 (39)	21 (58)*	0.07
Wheezing	3 (8)	1 (3)	6 (17)	10 (28)*	0.005

Data are presented as n (%) or median (interquartile range), unless otherwise stated. BMI: body mass index; Ig: immunoglobulin; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; ND: not determined. #: <5 cigarettes·day^{-1}; ¶: ⩾5 cigarettes·day^{-1}. Statistical analysis: Chi-squared test for frequencies, Dunnett's *post hoc* test for continuous variables; p-values obtained using Chi-squared test for trend for proportions or from one-way ANOVA for continuous variables. *: $p<0.05$ pair-wise comparison with nonsmokers.

TABLE 2 Biomarkers in nasal lavage fluid (NALF), serum and exhaled breath

	Nonsmokers	Passive smokers	Light smokers#	Heavy smokers¶	p-value
Markers in NALF					
Volume mL	3.65 (2.96–4.3)	3.8 (3.19–4.31)	3.53 (3.08–4.01)	3.91 (3.25–4.25)	0.71
Urea					
Concentration mg·L^{-1}	38.8 (32–57.7)	41.2 (29.3–50.4)	46.8 (30.6–62.6)	36.5 (28.4–42.6)	0.18
Recovered mg	0.15 (0.11–0.19)	0.15 (0.12–0.18)	0.15 (0.13–0.21)	0.13 (0.11–0.18)	0.33
Serum urea mg·mg^{-1}	0.17 (0.13–0.19)	0.17 (0.13–0.23)	0.19 (0.12–0.23)	0.17 (0.12–0.20)	0.94
CC16					
Concentration μg·L^{-1}	28.7 (11.5–85.4)	9.5 (2.8–50.8)	19.1 (9.5–54.2)	11.2 (3.6–26.3)*	0.050
Recovered ng	94 (45–313)	41 (11–174)	71 (37–172)	45 (15–100)*	0.04
Adjusted for urea ratio μg·L^{-1}	174 (101–533)	100 (24–307)	135 (58–243)	61 (24–155)*	0.02
Albumin					
Concentration mg·L^{-1}	8.8 (4.1–16.5)	6.3 (3.4–22.7)	9.7 (6.1–15.5)	5.8 (2.6–13.5)	0.62
Recovered μg	27.4 (16.6–45)	28.9 (12.3–71.7)	34.4 (18.8–55.2)	22.9 (9.9–53.2)	0.70
Adjusted for urea ratio mg·L^{-1}	46.2 (30.2–79.1)	39.9 (22.5–118.0)	48.28 (29.4–104.2)	32.73 (17.3–50.1)	0.55
Markers in exhaled breath					
NO in oral exhalate ppb	15.2 (11.3–24.6)	11.4 (7.4–20.1)	17.8 (11–28.8)	8.9 (6.7–11.9)*	<0.001
Markers in serum					
CC16					
Concentration μg·L^{-1}	8.6 (7.1–13.1)	9.7 (7.1–12)	9.7 (6.9–13.3)	8.6 (7–10.5)	0.90
Ratio CC16 to SP-D	0.12 (0.06–0.18)	0.12 (0.09–0.21)	0.12 (0.08–0.19)	0.11 (0.08–0.17)	0.21
SP-D concentration μg·L^{-1}	87 (55–112)	63 (50–96)	80 (53–110)	75 (59–111)	0.13
Creatinine mg·L^{-1}	0.91 (0.81–0.99)	0.84 (0.8–0.94)	0.96 (0.82–1.05)	0.89 (0.81–0.97)	0.62
Urea mg·L^{-1}	243 (204–301)	262 (212–288)	271 (244–295)	242 (186–257)	0.11

Data are presented as median (interquartile range), unless otherwise stated. CC16: Clara cell protein; NO: nitric oxide; SP: surfactant protein. #: <5 cigarettes·day^{-1}; ¶: ⩾5 cigarettes·day^{-1}. Statistical analysis: one-way ANOVA followed by Dunnett's *post hoc* test. *: p<0.05 compared with nonsmokers.

absolute amount of recovered CC16 or by adjusting the CC16 concentration for the plasma/NALF urea concentration ratio. Notably, passive smokers showed a systemic tendency to lower CC16 levels in NALF compared with nonsmokers, especially after adjustment for the plasma/NALF urea concentration ratio (Dunnett's *post hoc* test p=0.08). The most statistically significant difference, however, concerned the concentration of exhaled NO, which was noticeably reduced in the group of heavy smokers (median 42% lower than in nonsmokers). Concentrations of albumin in NALF and of CC16 and SP-D in serum, in contrast, did not vary between the different groups.

Multivariate analyses confirmed the decrease of NALF CC16 (normalised to urea ratio; slope -0.76, p=0.02) and of exhaled NO (slope -0.38, p=0.04) in the group of heavy smokers and the lack of change in the other markers measured in NALF or serum. Figures 1 and 2 illustrate the changes in the covariate-adjusted concentrations of CC16 in NALF, of exhaled NO, and

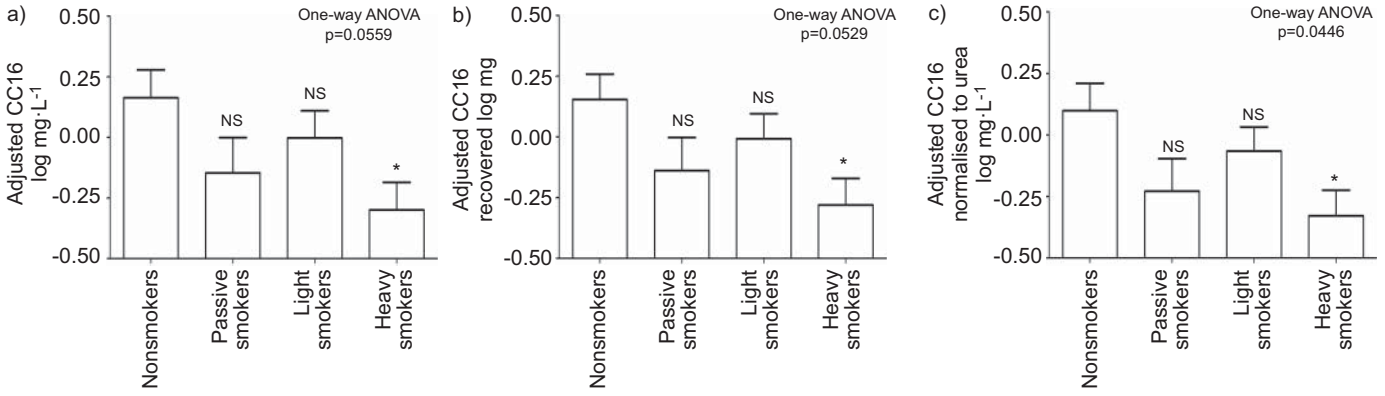


FIGURE 1. a) Adjusted Clara cell protein (CC16) level, b) CC16 amount recovered and c) CC16 adjusted for urea ratio in nasal lavage fluid. Data are presented as mean±SEM. Light smokers: <5 cigarettes·day^{-1}; heavy smokers: ⩾5 cigarettes·day^{-1}. a and b) CC16 adjusted for room ventilation, having a heating system with wood at home, having older siblings, asthma, bronchiolitis episode and frequent colds. c) CC16 adjusted for room ventilation, having a heating system with wood at home, having older siblings, asthma and frequent colds. Statistical analysis: one-way ANOVA followed by Dunnett's *post hoc* test. NS: nonsignificant; *: p<0.05 compared with nonsmokers.

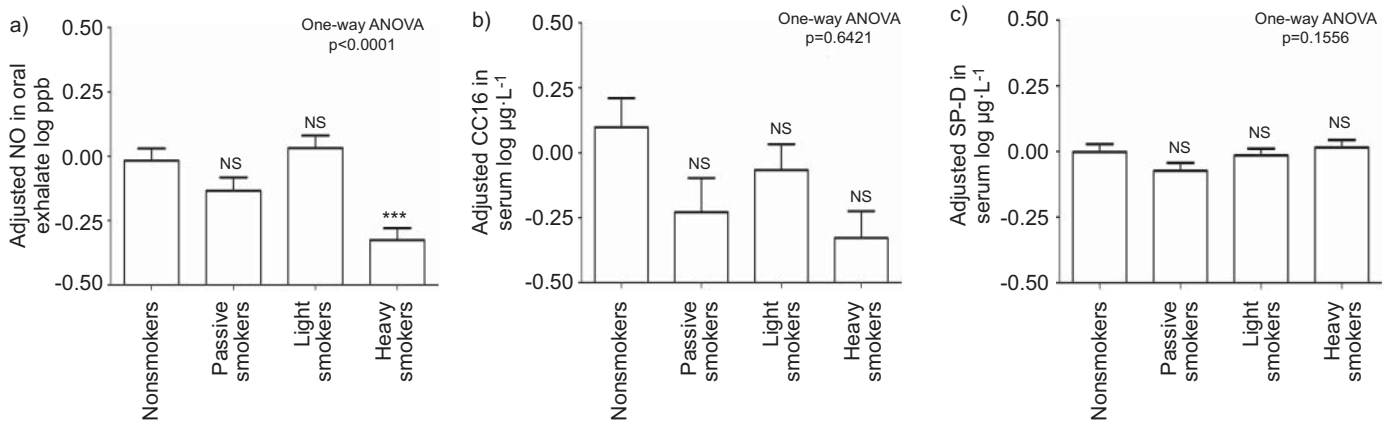


FIGURE 2. a) Adjusted nitric oxide (NO) levels in oral exhalate, b) adjusted Clara cell protein (CC16) levels in serum and c) adjusted surfactant protein (SP)-D levels in serum. Data are presented as mean±SEM. Light smokers: <5 cigarettes·day^{-1}; heavy smokers: ⩾5 cigarettes·day^{-1}. a) NO adjusted for use of air fresheners, having a heating system with wood at home, allergic rhinitis, bronchitis episode, frequent colds, immunoglobulin (Ig)E against aeroallergens and total IgE in serum. b) CC16 adjusted for age, body mass index, living in a rural area, use of bleach as a cleaning agent at home, having a heating system with wood, having older siblings, cumulated chlorinated pool attendance, asthma, bronchiolitis episode, time of sample collection and serum creatinine. c) SP-D adjusted for sex, day care attendance, parent with higher education, maternal smoking during pregnancy, room ventilation, use of bleach as a cleaning agent at home, having a heating system with wood, having a furred pet, asthma, frequent colds, hay fever, IgE against aeroallergens and total IgE in serum. Statistical analysis: one-way ANOVA followed by Dunnett's *post hoc test*. NS: nonsignificant; ***: p<0.001 compared with nonsmokers.

of CC16 and SP-D in serum. We also examined whether the decreased levels of exhaled NO and of CC16 in NALF in smokers correlated with the smoking history. There was a highly significant negative association between exhaled NO and the number of cigarettes smoked per day (fig. 3a). A negative association also emerged between CC16 in NALF and cigarette consumption but without reaching the level of statistical significance (fig. 3b). Similar associations were observed with the number of pack-years (results not shown). There was no apparent relationship between CC16 in NALF and exhaled NO, as evidenced by a Spearman correlation value of 0.16 and a p-value of 0.28. There were also no differences in the covariate-adjusted levels of CC16 and of exhaled NO between adolescents with or without respiratory symptoms (results not shown). No statistically significant interaction between smoking status and sex was detected in the tested models. As illustrated in figure 3, exhaled NO and CC16 in NALF decreased rather similarly in males and females (exhaled NO: females r= -0.54, p=0.0001, males r= -0.50, p=0.008; CC16 in NALF: females r= -0.26, p=0.16, males r= -0.16, p=0.43).

DISCUSSION

The concentrations of CC16 in NALF and of NO in exhaled air were decreased in adolescents smoking ⩾5 cigarettes·day^{-1}. The low levels of these biomarkers persisted after adjustment for potential confounders and were correlated with the cigarette consumption. Quite remarkably, these biomarker changes occurred at a level of cumulative smoking that was about one order of magnitude lower than reported so far in adult smokers [15]. We found no change in the serum levels of CC16 and SP-D, which suggests that the deep lung epithelium had not yet been affected at this stage of smoking. This is really no surprise given the very low cumulative smoking of our adolescent study participants in comparison with previous studies reporting altered serum levels of these lung biomarkers [12, 13, 27, 28].

None of the studied biomarkers showed significant changes among adolescents who were light smokers or passive smokers, although there was a tendency of CC16 in NALF to decrease with passive smoking. This lack of biomarker response to passive smoking might appear inconsistent with the wealth of data, including those in the present study, showing that environmental tobacco smoke increases the risks of respiratory symptoms and other ailments [29]. The explanation for this discrepancy probably lies in the fact that studied biomarkers reflect chronic damage to the airways, which might require much higher cumulative doses of smoking than do respiratory symptoms. This is probably the reason why we could not detect any association between biomarker levels and respiratory symptoms in any of the groups exposed to cigarette smoke.

Our findings necessarily raise the question of the pathological significance of these early decreases of exhaled NO and of CC16 in NALF of smoking adolescents. Regarding exhaled NO, KHARITONOV *et al.* [15] have suggested that the reduced levels of this biomarker in smokers probably result from changes occurring in the upper respiratory tract. The authors proposed several mechanisms by which cigarette smoke might lower NO concentrations in exhaled breath: a downregulation of the NO synthase due to the NO present in the cigarette smoke itself, an inhibitory effect of CO which is also present in cigarette smoke, or else an inhibition of bacterial growth [15]. Whatever the exact mechanism, it is important to consider that NO plays an important role in the respiratory tract, in particular in the regulation of pulmonary and airway blood flow and the non-specific defence mechanisms [30]. This suggests that a chronic reduction of such a mediator is likely to have detrimental effects. The cigarette smoke-induced changes in exhaled NO were only observed in the group of heavy smokers and not in the groups of light smokers or passive smokers, meaning that these effects require a certain smoke dose to be elicited. A similar conclusion was drawn by GABRIELE *et al.* [16] when they found decreased

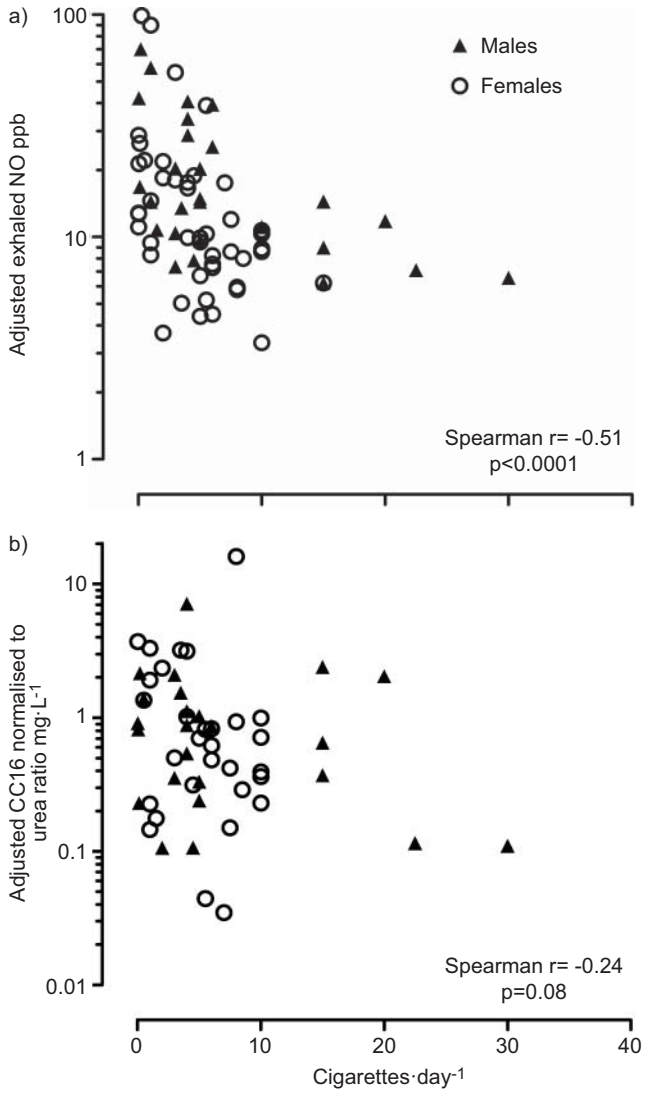


FIGURE 3. Relationships between daily cigarette consumption and epithelial biomarkers in males and females. a) Nitric oxide (NO) in exhaled air, b) Clara cell protein (CC16) normalised to urea in nasal lavage fluid. a) NO adjusted for use of air fresheners, having a heating system with wood at home, allergic rhinitis, bronchitis episode, frequent colds, immunoglobulin (Ig)E against aeroallergens and total IgE in serum. b) CC16 adjusted for room ventilation, having a heating system with wood at home, having older siblings, asthma and frequent colds. Relationships (both sexes together) were assessed using the Spearman nonparametric correlation test.

exhaled NO levels in infants exposed pre- and post-natally to tobacco smoke but not in never-exposed infants or in infants exposed to tobacco smoke only after birth.

It has been known a long time that the Clara cell is a sensitive target of cigarette smoke. Several studies have shown that cigarette smoking reduces the number of Clara cells and thereby the concentrations of CC16 in bronchoalveolar lavage, induced sputum and serum. CC16 is also a mediator that appears to have important regulatory and anti-inflammatory functions in the respiratory tract [4]. Moreover, Clara cells have the capability to differentiate into mucus-secreting cells [31], thus contributing to the evolution towards COPD stage 0, *i.e.* ''at risk'' according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [32]. Even if the exact role of CC16 at the level of the nasal cavity has not been elucidated, it appears reasonable to assume that cigarette smoke can cause similar epithelial damage in the upper airways to that in the lower airways. This possibility is supported, for instance, by the study of HADAR *et al.* [33], which describes goblet cell hyperplasia and thicker epithelium in the nasal mucosa of smokers.

A possible limitation of our study concerns the assessment of cigarette smoke exposure. Findings reported here were made in an add-on to a study initially designed to assess the impact of the environment on the health of adolescents. Although exposure to tobacco smoke was an important risk factor considered in our study, we did not measure exposure biomarkers such as serum or urinary cotinine, which anyway reflect only the recent exposure to tobacco smoke. However, to achieve the most objective assessment possible, we used the smoking habits reported by the adolescents themselves during a personal interview rather than those provided in the questionnaire filled by the parents. The fact that the main study was not focused on the effect of smoking might be more an advantage than an inconvenience. Our participants were blinded to the tested hypothesis, which allowed us to minimise the participation and response biases, which are important in this type of investigation. The reliability of our data about smoking habits is supported by the very significant dose–response relationships that emerged between exhaled NO and the daily or cumulative cigarette consumption.

In conclusion, the current biomarker study on adolescents shows that tobacco smoke can cause early changes in the airways as evidenced by decreased levels of CC16 in NALF and of NO in exhaled air. Quite remarkably, these effects are already apparent at cumulative cigarette consumption levels well below 1 pack-yr. These airway alterations in adolescents starting to smoke refute the common belief that tobacco smoke causes adverse effects occurring only later during adult life.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for E. Van Miert can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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Respiratory Effects Associated With Wood Fuel Use: A Cross-Sectional Biomarker Study Among Adolescents

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Summary. The use of wood as heating and cooking fuel can result in elevated levels of indoor air pollution, but to what extent this is related to respiratory diseases and allergies is still inconclusive. Here, we report a cross-sectional study among 744 school adolescents (median age 15 years) using as main outcomes respiratory symptoms and diseases, exhaled nitric oxide, total and aeroallergen-specific IgE in serum, and two epithelial biomarkers in nasal lavage fluid (NALF) or serum, that is, Clara cell protein (CC16) and surfactant-associated protein D (SPD). Information about the wood fuel use and potential confounders was collected via a personal interview of the adolescent and a questionnaire filled out by the parents. Two approaches were used to limit the possible influence of confounders, that is, multivariate analysis using the complete study population or pairwise analysis of matched sub-populations obtained using an automated procedure. Wood fuel use was associated with a decrease of CC16 and an increase of SPD in serum, which resulted in a decreased serum CC16/SPD ratio (median −9%, $P = 0.001$). No consistent differences were observed for the biomarkers measured in exhaled breath or NALF. Wood fuel use was also associated with increased odds for asthma [odds ratio (OR) 2.2, 95% CI: 1.1–4.4, $P = 0.02$], hay fever (OR = 2.4, 95% CI: 1.4–4.3, $P = 0.002$), and sensitization against pollen allergens (OR = 2.1, 95% CI: 1.3–3.4, $P = 0.002$). The risks of respiratory tract infections, self-reported symptoms, and sensitization against house-dust mite were not increased by wood fuel use. The increased risks of asthma, hay fever and aeroallergen sensitization, and the changes of lung-specific biomarkers consistently pointed towards respiratory effects associated with the use of wood fuel. **Pediatr Pulmonol. 2012; 47:358–366.** © 2011 Wiley Periodicals, Inc.

Key words: indoor pollution; lung biomarkers; respiratory disease; sensitization; wood fuel use; wood smoke.

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INTRODUCTION

Evidence is increasing that indoor pollution plays an important role in the development of respiratory diseases, especially during childhood.[1] A number of risk factors have already been identified, such as volatile organic compounds,[2] ultra-fine particles,[3] house dust allergens,[4] environmental tobacco smoke,[5] biomass burning smoke,[6] and chlorination products in swimming pools.[7] The use of wood as heating and cooking fuel can result in elevated levels of indoor air pollution and is considered to be an important public health problem in the developing world.[8] Illustrative for the currently increased attention to wood smoke are the recent classification by the International Agency for Research on Cancer of indoor emissions from biomass fuel (primarily wood) as a probable human carcinogen (group 2A) and the recent report by Hosgood et al. confirming the positive association between in-home wood use and the lung cancer risk.[9,10] Epton et al.[11] did not detect a significant effect of ambient wood smoke particulate air pollution on lung function of healthy school-aged students but they observed associations with respiratory symptoms. In a recent review, Po et al.[12] reported significant associations between solid biomass fuel exposure and the risk of acute respiratory infection in children [odds ratio (OR) 3.53, 95% CI: 1.94–6.43] but these authors found no association with asthma. Barry et al.[13] reported that cooking indoors with wood and coal for more than 6 months significantly increased the

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odds of current asthma (OR = 2.3, 95% CI: 1.1–5.0), whereas no effect was seen with history of heating indoors with wood and coal (OR = 0.8, 95% CI: 0.4–1.8). In another recent study, Nguyen et al.[14] reported a statistically significant negative association between asthma and the use of a wood-burning stove. Maier et al.[15] did not observe an association between the use of a wood stove and asthma or wheezing. On the other hand, in the experimental study by Samuelsen et al.,[16] wood smoke exhibited an adjuvant-like activity in allergic sensitization.

In recent years, there have been significant advances in the development of non- or less-invasive tools to monitor airways damage or inflammation. The amount of data supporting nitric oxide (NO) in exhaled air as a non-invasive marker of airways inflammation in asthma and allergic diseases has been steadily growing.[17] Also, Clara cell-specific protein (CC16) and surfactant protein D (SPD), which is mainly produced by the type II pneumocytes, have been shown to be sensitive biomarkers of epithelial damage in lower airways.[18] The measurement of biomarkers in nasal lavage fluid (NALF) is another non-invasive approach, which is receiving increased interest because it allows to detect nasal epithelial changes relevant to the deeper lung.[19] Barregard et al.[20] were among the first to use such biomarkers to assess the respiratory effects of wood smoke. In a controlled human exposure study, these authors showed that a single exposure to wood smoke causes increases of NO in exhaled air and of CC16 in serum. The increased level of NO is indicative of an inflammation at the distal part of the airways, while the increased CC16 concentration in serum is the reflection of an increased permeability of the air–blood barrier.

Using the large database which has been generated by our laboratory during a cross-sectional survey of more than 800 adolescents, we investigated whether wood fuel use was associated with respiratory diseases, sensitization to aeroallergens, self-reported symptoms, or changes of epithelial biomarkers in serum, exhaled breath and NALF.

ABBREVIATIONS:

CC16	Clara cell protein CC16
CI	confidence interval
FEV_1	forced expiratory volume in 1 sec
FVC	forced vital capacity
IgE	immunoglobulin E
NALF	nasal lavage fluid
NO	nitric oxide
OR	odds ratio
SPD	surfactant protein D

METHODS

Study Population

The database on adolescents was generated according to a study protocol which was approved by the Ethics committee of the Faculty of Medicine of the Catholic University of Louvain and which complied with all applicable requirements of international regulations. The adolescents attended school in the cities of Louvain-la-Neuve, Bastogne or Lessines, 3 small-size (<30,000 inhabitants) cities in the French speaking part of Belgium. The first 2 cities are located in rural regions, whereas the third one is part of a larger urban region. One school per city was selected, and of the 1,200 students (3rd or 4th grade of secondary school) who were contacted, 1,137 (94.8%) returned the questionnaire. Among them, 847 had written the agreement from their parents to participate in the study, resulting in an overall participation rate of 71.4%. As active smoking could act as a major confounder, all students who had declared during the personal interview to be active smokers were excluded from the study population. When excluding the active smokers (n = 84) and the adolescents lacking a properly filled-out questionnaire (n = 19), a total of 744 adolescents were retained for further analysis.

Questionnaire

The adolescents' parents were asked to complete a questionnaire about the personal or familial characteristics, the in-house and out-house environment, medical characteristics and antecedents, and self-reported symptoms. The information about the personal and familial characteristics comprised gender, ethnicity (not included in further analyses because only 3% were of non-Caucasian origin), age, birth weight, smoking status of mother during pregnancy, a parent with allergy or asthma, having older siblings, day care attendance, and having a parent with bachelor level education or higher (as a marker for social status). Investigated characteristics of the out-house environment where living in a rural area and the cumulated attendance to chlorinated pools. The in-house environment was characterized by living in a house built after 1950, having a furred pet, low ventilation of the bedroom (one or less times per week), presence of molds on walls, the use of bleach as cleaning agent, smoking parents (i.e., being exposed to environmental tobacco smoke), use of air fresheners, and having a heating system with wood (e.g., fireplace, stove). Medical characteristics were doctor-diagnosed asthma, hay fever (seasonal allergic rhinitis), and allergic rhinitis (non-seasonal allergic rhinitis). Information about medical antecedents concerned the past occurrence of lower respiratory tract

infections (pneumonia, bronchitis, bronchiolitis) and frequent colds (>4×/year).

Examination of Students

The adolescents participating in the study were examined in their school between March 7 and May 15, 2006. Examinations took place between 09:00 and 15:00. For each test or sample collection, the time was recorded to adjust for the possible diurnal variations in the biomarker levels. The examination started with an interview inquiring about the respiratory symptoms during the past 12 months (wheezing, cough crisis, chest tightness, and shortness of breath), the smoking habits, and the medication consumption. Additionally, the answers to the main questions of the questionnaire, such as those relating to the characteristics of the home environment, were reviewed with the study participant. In case of contradictions, the answer obtained during the interview outweighed the answer from the questionnaire. Next, the adolescents underwent a series of tests including the measurement of height and weight, NO in exhaled breath and lung function, and the collection of blood and NALF samples. The forced expiratory volume in 1 sec (FEV_1), and the forced vital capacity (FVC) were measured with a Medikro spirometer (Spiro2000 v1.7.3, Medikro OY, Finland) according to the American Thoracic Society Standards.[21] The concentration of NO in exhaled air was determined with the NIOX^{TM} analyzer (Aerocrine AB, Solna, Sweden) according to the guidelines of the American Thoracic Society.[22] The sample of venous blood was collected on a dry tube, allowed to clot overnight at 4°C, and then centrifuged at 2,000*g* for 10 min. Serum was decanted and stored at −18°C until biomarker analysis. NALF samples were collected from both nostrils. Participants were asked to sit down, bend forward, and put their heads down. Two and a half milliliters of sterile physiological saline at 37°C were instilled into each nostril by a disposable tip connected to a peristaltic pump. After 10 sec, students were asked to lift their head and the lavage fluid was collected using a small funnel. The NALF samples were stored at −20°C until evaluation.

Analyses

Total and aeroallergen-specific IgE concentrations in serum were determined using the Immulite® IgE kit (Diagnostic Products Company, Los Angeles, CA). The presence of IgE against the following allergens was verified: house-dust mite, cat epithelium, dog dander, molds, tree pollen, grass pollen, and herbaceous pollen mixture (weeds). Sensitization against specific aeroallergens was defined as a serum concentration of specific IgE >0.35 kIU/L. The CC16 concentration in serum and NALF was determined by latex immunoassay using a rabbit anti-CC16 antibody (Dakopatts, Glostrup, Denmark) and CC16 standards purified in our laboratory.[23] The serum concentration of SPD was measured using the commercially available SPD EIA kit (Code No YSE-7744; Yamasa Corporation, Chiba, Japan). Results from the NALF samples were available for a total of 419 adolescents. Care was taken to include only the results from adolescents in the study who did not had a cold 2 weeks prior to the sample collection as this could possibly bias the interpretation. Concentrations of biomarkers in NALF were adjusted for the variable dilution of the recovered epithelial lining fluid either by calculating the absolute amount of recovered protein or by adjusting the concentration in NALF with the plasma/NALF concentration ratio of urea.[24,25] These adjustments were made for each nostril separately and then the mean value was calculated and used for the statistical analyses.

Statistical Analyses

Bivariate and multivariate analyses were performed using R.[26–28] Graphs and statistics on adjusted concentrations were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Statistical procedures on continuous variables were performed after log-transformation. The threshold for statistical significance was set at $P < 0.05$. Comparisons of continuous variables between the non-exposed and exposed adolescents were performed using the *t*-test. To eliminate the influence of confounding factors, two approaches were used, that is, matching prior to statistical testing and multivariate analyses. In the first approach, pairwise statistical tests were performed after matching of adolescents exposed to wood (smoke, n = 226) to a comparable population of non-exposed adolescents (n = 226) by means of the automated matching procedure "optmatch[29]" using the characteristics: "gender," "age," "parental allergy or asthma," "parental higher education," "living in a rural area," and "living in a house built after 1950." Multivariate analyses were performed on the total study population (744 adolescents). Adjusted OR were obtained by means of the generalized linear modeling using the binomial distribution, whereas multivariate models for biomarkers in NALF, blood and exhaled air were built via the same approach but using the Gaussian distribution. Initial models were defined associating each biomarker with wood fuel use and with age, gender, body mass index (BMI), birth weight, total serum IgE concentration (except for sensitization outcomes), time of sample collection (biomarkers only), the social and medical characteristics, and the variables reflecting the in- and out-house environment quality.

For serum markers, also the serum creatinine concentration was included in the initial model to account for the renal function. The obtained models were optimized using a backward stepwise algorithm using the Akaike information criterion (AIC, R-function: ''step'').

RESULTS

The characteristics of the sub-populations with or without matching are shown in Table 1. When comparing the unmatched sub-populations, adolescents living in a house heated with wood did not differ from their peers with respect to gender, age, older siblings, parental history of asthma, and allergy and chlorinated pool attendance. The group had a lower socio-economic status as reflected by the parental education and fewer had attended a day care center during infancy. Not surprisingly, houses heated with wood were more often old houses localized in a rural area. When comparing the matched sub-populations, none of the above-mentioned significant differences was present anymore.

Table 2 displays the respiratory health characteristics of adolescents with or without wood fuel use. The self-reported symptoms, allergic rhinitis, and the respiratory tract infections did not differ meaningfully between the two groups, irrespective whether the sub-populations were matched or not. Multivariate analysis confirmed the above-mentioned absence of findings. Wood fuel use was not retained in any of the optimized models with a P-value <0.05. On the other hand, the wood fuel use group showed increased prevalence of asthma and hay fever. Atopic asthma, that is, doctor-diagnosed asthma with a concurrent aeroallergen sensitization, was consistently associated with wood fuel use. The OR for non-atopic asthma, that is, doctor-diagnosed asthma without concurrent aeroallergen sensitization, was similar to that of atopic asthma, but statistical significance was not reached. Adolescents living in a house with a heating system with wood consistently showed higher frequencies of sensitization against pollen (sensitization against at least one of the pollen antigens tested) and against pollen from weeds, trees, and grass, but not against house dust mite or all aeroallergens (sensitization against at least one of the tested allergens).

The results of the lung function measurements and determinations of biomarkers in exhaled air, NALF, and serum are provided in Table 3. Wood fuel use was not associated with changes in lung function measures or in the levels of exhaled NO. There were also no differences in the levels of CC16 or urea in NALF. Pairwise analyses on the entire study population showed that albumin in NALF was significantly higher in the group with wood fuel use. Multivariate analysis confirmed

TABLE 1— Characterization of the Sub-Populations With or Without Wood Fuel Use

	Wood fuel use all study subjects			Wood fuel use matched study subjects		
	No	Yes		No	Yes	
	n = 514	n = 230	*P*-value	n = 226	n = 226	*P*-value
Personal/family characteristics						
Female gender, n (%)	289 (56)	124 (54)	0.56	125 (55)	122 (54)	0.78
Age, median (IQR, years)	15.3 (14.8–16.0)	15.2 (14.7–15.8)	0.12	15.2 (14.7–15.8)	15.2 (14.7–15.8)	0.61
BMI, median (IQR), kg/m^2	20.2 (18.7–22.2)	19.8 (18.5–21.9)	0.19	20.2 (18.7–22.5)	19.8 (18.5–22.0)	0.31
Birth weight, median (IQR), kg	3.32 (3.00–3.60)	3.32 (3.00–3.70)	0.77	3.32 (3.00–3.62)	3.32 (3.00–3.70)	0.68
Parental allergy or asthma, n (%)	229 (45)	96 (42)	0.48	95 (42)	94 (42)	0.92
High parental education, n (%)	388 (75)	154 (67)	0.02	155 (69)	151 (67)	0.69
Maternal smoking during pregnancy, n (%)	62 (12)	22 (10)	0.32	29 (13)	22 (10)	0.30
Day care attendance, n (%)	258 (50)	91 (40)	0.01	108 (48)	89 (39)	0.07
Having older siblings, n (%)	298 (58)	133 (58)	0.97	122 (54)	129 (57)	0.51
Environment						
House in rural area, n (%)	332 (65)	206 (90)	2.E−12	204 (90)	204 (90)	1.00
Chlorinated pool attendance (>500 hr), n (%)	211 (41)	111 (48)	0.07	92 (41)	108 (48)	0.13
House built after 1950, n (%)	337 (66)	134 (59)	0.04	131 (58)	133 (59)	0.85
Having a furred pet, n (%)	384 (75)	180 (78)	0.30	180 (80)	177 (78)	0.73
Low room ventilation, n (%)	98 (19)	32 (14)	0.09	46 (20)	31 (14)	0.06
Molds on walls at home, n (%)	35 (7)	13 (6)	0.55	16 (7)	12 (5)	0.44
Cleaning with bleach, n (%)	141 (27)	50 (22)	0.10	58 (26)	49 (22)	0.32
Use of air fresheners, n (%)	133 (26)	56 (24)	0.66	56 (25)	55 (24)	0.91
Environmental tobacco smoke, n (%)	182 (35)	69 (30)	0.15	84 (37)	67 (30)	0.09

Data are provided for the sub-populations comprising all study subjects and for the matched sub-populations. Number of cases (%) or median (interquartile range), P-values from chi-squared test (frequencies), or t-test (continuous variables), comparisons relative to corresponding group without the exposure to wood smoke.

TABLE 2— Frequencies of Respiratory Symptoms and Diseases and Aeroallergen-Specific Sensitization With Respect to Wood Fuel Use, and the Associated Crude and Adjusted Odds Ratios [OR, Provided as Odds Ratio (95% Confidence Interval)]

	Wood fuel use all study subjects					Wood fuel use matched study subjects		
	No (n = 514)	Yes (n = 230)	OR	Adj. OR		No (n = 226)	Yes (n = 226)	OR
	n (%)	n (%)	(95% CI)	(95% CI)	*P*-value	n (%)	n (%)	(95%CI)
Respiratory symptoms								
Wheezing	56 (11)	27 (12)	1.1 (0.7–1.8)			25 (11)	27 (12)	1.1 (0.6–2.0)
Chest tightness	20 (4)	7 (3)	0.8 (0.3–1.8)			8 (4)	7 (3)	0.9 (0.3–2.5)
Cough crisis	73 (14)	35 (15)	1.1 (0.7–1.7)			34 (15)	34 (15)	1.0 (0.6–1.7)
Nasal problems	225 (44)	96 (42)	0.9 (0.7–1.3)			97 (43)	93 (41)	0.9 (0.6–1.4)
Shortness breath	35 (7)	11 (5)	0.7 (0.3–1.4)			17 (8)	11 (5)	0.6 (0.3–1.4)
Respiratory diseases								
Doctor-diagnosed asthma	44 (9)	31 (13)	1.7 (1.0–2.7)	1.9 (1.1–3.4)	0.02	14 (6)	31 (14)	2.4 (1.3–4.8)
Atopic asthma	33 (6)	22 (10)	1.5 (0.9–2.7)	1.9 (1.0–3.8)	0.049	9 (4)	22 (10)	2.6 (1.2–6.1)
Non-atopic asthma	11 (2)	9 (4)	1.9 (0.7–4.6)			5 (2)	9 (4)	1.8 (0.6–6.1)
Allergic rhinitis	77 (15)	46 (20)	1.4 (0.9–2.1)	1.4 (0.9–2.3)	0.10	29 (13)	45 (20)	1.7 (1.0–2.8)
Hay fever	64 (12)	54 (24)	2.2 (1.4–3.2)	2.3 (1.4–3.6)	<0.001	26 (12)	53 (23)	2.3 (1.4–4.0)
Pneumonia	80 (16)	28 (12)	0.8 (0.5–1.2)	0.7 (0.4–1.1)	0.10	32 (14)	27 (12)	0.8 (0.5–1.4)
Bronchitis	224 (44)	103 (45)	1.1 (0.8–1.4)			100 (44)	101 (45)	1.0 (0.7–1.5)
Bronchiolitis	70 (14)	33 (14)	1.1 (0.7–1.7)			30 (13)	32 (14)	1.1 (0.6–1.9)
Frequent colds (≥4/year)	153 (30)	77 (34)	1.2 (0.9–1.7)			69 (31)	75 (33)	1.1 (0.8–1.7)
IgE test (positive: >0.35 kIU)								
Aeroallergens (at least 1)	191 (37)	93 (41)	1.2 (0.8–1.6)			78 (35)	92 (41)	1.3 (0.9–1.9)
Pollen (at least 1)	92 (18)	65 (28)	1.8 (1.3–2.6)	1.9 (1.3–2.8)	0.001	36 (16)	65 (29)	2.1 (1.3–3.4)
Weed pollen	23 (4)	20 (9)	1.2 (0.9–1.7)	1.9 (1.0–3.6)	0.06	11 (5)	20 (9)	1.9 (0.9–4.2)
Tree pollen	40 (8)	33 (14)	2.0 (1.1–3.8)	1.8 (1.1–3.0)	0.03	16 (7)	33 (15)	2.2 (1.2–4.3)
Grass pollen	75 (15)	56 (24)	2.0 (1.2–3.3)	2.0 (1.3–3.0)	0.001	23 (10)	56 (25)	2.9 (1.7–5.0)
House dust mites	132 (26)	68 (30)	1.9 (1.3–2.8)			55 (24)	67 (30)	1.3 (0.9–2.0)

Adjusted odds ratios are only provided for total study population and if the exposure to wood smoke was retained in the optimized model, *P*-value given for adjusted OR.

these findings. Wood fuel use was significantly associated with the albumin levels in NALF (slope factors of 0.19, 0.24, and 0.021 and *P*-values of 0.08, 0.02, and 0.04 for the albumin concentration, the albumin concentration adjusted for plasma/NALF urea ratio and the albumin recovery, respectively). When comparing the matched sub-populations, no differences for albumin in NALF could be detected. The concentrations of IgE, creatinine, and urea in serum did not show statistically significant differences. When comparing the non-matched sub-populations, lower levels of CC16 and higher levels of SPD were observed in the adolescents with wood fuel use. These differences persisted after adjustment for renal function using the creatinine concentration. As a consequence, the CC16 to SPD ratio in serum, integrating the changes of both biomarkers, was significantly decreased (median −10%). Multivariate analysis confirmed the difference of the CC16 levels in serum, but only a trend for SPD remained with slope factors of −0.07 and 0.06 (log-transformed) and *P*-values of 0.02 and 0.14, respectively. In the multivariate model of the CC16 to SPD ratio, wood fuel use had a slope factor of −0.13 and *P*-value of 0.01. When comparing the matched sub-populations, a similar pattern was observed. A small but statistically significant decrease of serum CC16 (median −3.5%), and a weak trend towards increased levels of serum SPD (median +4.5%). The CC16/SPD ratio in serum was statistically significantly lower (−9%) in the sub-population with wood fuel use.

As discussed above, wood fuel use showed significant and consistent associations with doctor-diagnosed asthma, hay fever, sensitization against pollen, and the CC16 to SPD ratio in serum. In order to further explore these relationships, we included multiplicative interaction terms combining wood fuel use with doctor-diagnosed asthma, hay fever, or sensitization against pollen in the initial multivariate model of the CC16 to SPD (log-transformed). Except for wood fuel use (slope −0.13, *P*-value = 0.007) and sensitization against pollen (slope 0.08, *P*-value = 0.13), none of the above-mentioned variables or interaction terms was retained in the model after optimization. Figure 1 illustrates this in another way by showing the CC16/SPD ratio in serum for the matched sub-populations with respect to wood fuel use and asthma (a), hay fever (b), or

TABLE 3— Lung Function Parameters and Biomarkers in Exhaled Air, Nasal Lavage Fluid, and Serum of Sub-Populations With or Without Wood Fuel Use

	Wood fuel use all study subjects			Wood fuel use matched study subjects		
	No (n = 514)	Yes (n = 230)	*P*-value	No (n = 226)	Yes (n = 226)	*P*-value
Lung function measurements						
FEV_1 % pred. values	100 (92–110)	99 (92–108)	0.44	98.5 (92–109)	99.5 (92–108.8)	0.81
FVC % pred. values	89 (80–97)	88 (79–97)	0.47	88 (80–95)	88 (79–97)	0.79
FEV_1/FVC ratio	1.15 (1.1–1.19)	1.16 (1.11–1.19)	0.96	1.15 (1.1–1.18)	1.16 (1.11–1.19)	0.97
Exhaled breath						
NO (ppb)	13.2 (9.4–19.9)	13.2 (9.9–21.0)	0.58	12.4 (8.9–21.7)	13 (9.7–21.0)	0.80
Nasal lavage fluid						
Volume (ml)	3.71 (3.03–4.09)	3.64 (3.04–4.18)	0.78	3.74 (3.17–4.08)	3.65 (3.1–4.19)	0.72
CC16						
Concentration (μg/L)	21.3 (8.4–71.4)	26.5 (9.0–72.9)	0.43	22 (8.1–68.8)	26.5 (8.9–68.8)	0.52
Adjusted for plasma/NALF urea ratio (μg/L)	3.7 (1.2–15.9)	4.3 (1.2–13.3)	0.43	4.3 (1.2–14.4)	4.3 (1.1–12.9)	0.60
Recovery (ng)	78 (31–206)	95 (32–233)	0.38	82 (30–193)	95 (31–228)	0.52
Albumin						
Concentration (mg/L)	8.6 (4.1–15.8)	10.2 (5.8–19.1)	0.02	10.3 (4.9–18.7)	10.0 (5.8–19.4)	0.32
Adjusted for plasma/NALF urea ratio (μg/L)	1.40 (0.60–3.38)	1.85 (0.91–3.59)	0.03	1.70 (0.72–3.60)	1.81 (0.91–3.63)	0.46
Recovery (μg)	28.1 (15–50.7)	36.7 (21–64.4)	0.01	34.6 (17.3–65.7)	36.7 (20.9–66.3)	0.35
Urea						
Concentration (mg/L)	40.7 (31.5–53.1)	43.3 (32.3–58.5)	0.27	43.7 (32.5–55.2)	43.3 (32.9–58)	0.95
Recovery (mg)	0.14 (0.11–0.18)	0.15 (0.12–0.19)	0.16	0.15 (0.12–0.2)	0.15 (0.12–0.19)	0.76
Serum						
Urea (mg/L)	248 (213–291)	257 (214–309)	0.23	259 (218–299)	258 (215–310)	0.72
Creatinine (mg/L)	8.7 (8.0–9.7)	0.88 (0.79–0.98)	0.54	0.87 (0.80–0.95)	0.88 (0.79–0.98)	0.74
Total IgE (kIU/L)	47 (17–145)	63 (20–170)	0.38	46 (16–132)	63 (21–166)	0.24
SPD (μg/L)	87 (63–120)	91 (63–137)	0.02	88 (64–120)	92 (63–138)	0.12
SPD/creatinine ratio ($\times 10^{-3}$)	9.6 (6.8–14.0)	10.3 (7.2–16.7)	0.02	10.1 (7.1–14.2)	10.4 (7.3–16.8)	0.17
CC16 (μg/L)	8.9 (6.7–11.9)	8.4 (6.3–10.5)	0.01	8.7 (6.7–11.9)	8.4 (6.3–10.5)	0.03
CC16/creatinine ratio ($\times 10^{-3}$)	1.00 (0.74–1.35)	0.96 (0.71–1.18)	0.02	1.01 (0.75–1.37)	0.96 (0.71–1.18)	0.01
CC16/SPD ratio ($\times 10^{-3}$)	102 (67–152)	92 (52–136)	0.001	100 (69–142)	91 (52–135)	0.01

Data presented as medians (inter-quartile range), statistics: unpaired *t*-test on log-transformed data.

sensitization against pollen (c). Two-way analysis yielded three similar graphs. The serum CC16/SPD ratio was consistently and significantly lower in the groups with wood fuel use, whereas the other investigated variables showed no association with the CC16 to SPD ratio. Similar to the multivariate analysis, the two-way analyses of the matched study populations did not detect a statistically significant interaction between 1 of the 3 variables and wood fuel use.

DISCUSSION

In this study, wood fuel use was associated with a decreased level of CC16 and an increased level of SPD in serum, and, consequently, a decreased serum CC16/SPD ratio. Wood fuel use was also associated with increased risks of asthma, hay fever, and sensitization against pollen allergens. Even when limiting the effects of potential confounders by using an automated matching of the sub-populations or multivariate models with step-wise optimizations, the above-mentioned associations were consistently observed. Although the albumin levels in NALF were significantly increased when considering the whole study population and an increased extravasation of albumin into the airways is a known effect of toxic lung injury,[30] this effect was not retained as a key finding of our study on the basis that it was completely abolished by the automated matching of study subjects.

It is worth noting that the changes described in this study resemble those reported by Robin et al.,[31] who observed in smokers a decrease of CC16 and an increase of surfactant protein B. Whereas the decrease of CC16 in serum could reflect a decreased production of the Clara cells due to the irritation/cytotoxicity of smoke, the increase of surfactant proteins in serum might be explained by an unchanged production by the type II pneumocytes accompanied by an increased permeability of the lung epithelial barrier.[18] The decrease of the CC16 levels as observed in the current study (median −6%) is not as strong as Robin et al.[31] observed in smokers (median −28%). This might be

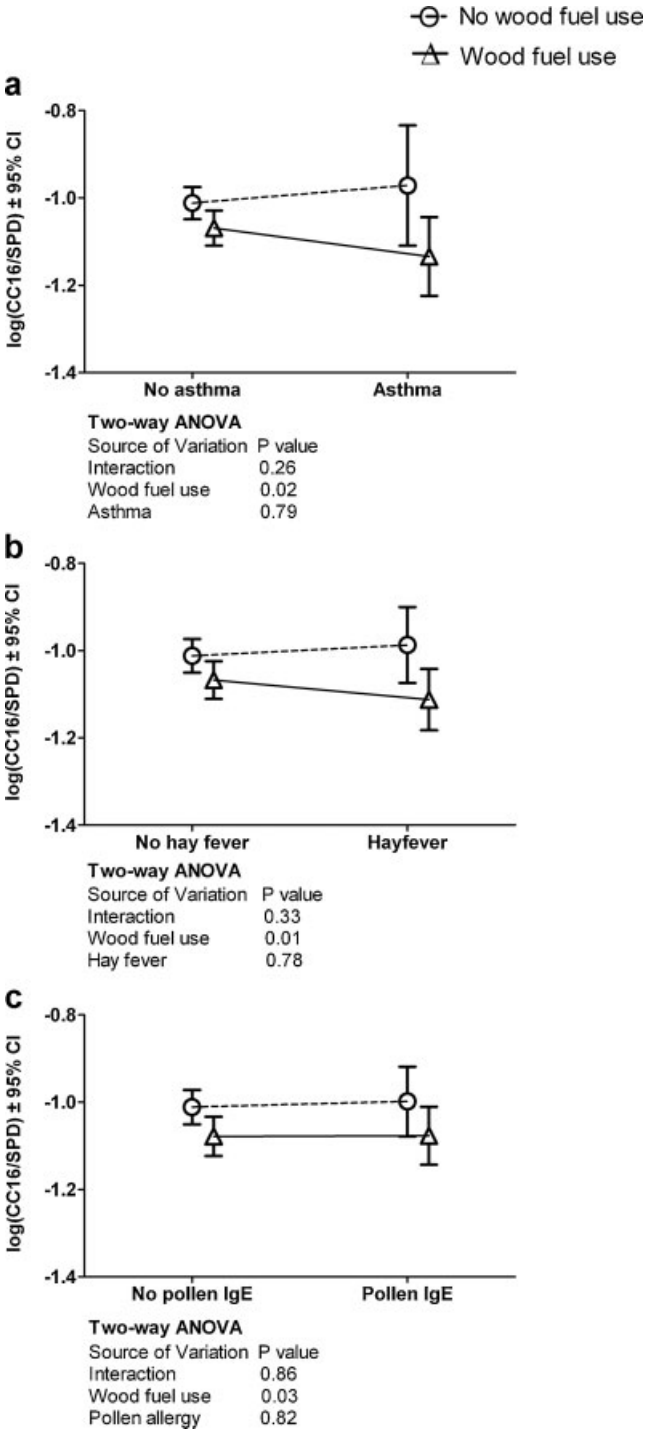


Fig. 1. Two-way analysis of the matched dataset investigating the relationships between the CC16 to SPD ratios in serum and asthma (a), hay fever (b), and sensitization against pollen (c) with or without concurrent wood fuel use.

explained by differences in study populations and in the exposure levels. Whereas the current study investigated non-smoking adolescents with a seasonal exposure to "wood fuel use," Robin et al.[31] investigated adult smokers with a median cumulative pack-years of 14.9. Moreover, even though exposure to combustion products is also involved in wood fuel use, a significant difference between the levels of exposure to hazardous chemicals in both studies is more than fair assumption.

The apparent paradox with the elevated serum levels of CC16 in the study by Barregard et al.[20] can be explained by the fact that wood smoke exposure in that study was acute and that the driving mechanism in that case was probably lung hyperpermeability and not the reduced production of CC16. As both wood smoke and tobacco smoke originate from the incomplete combustion of biomaterials, common mechanisms might underlie the observed changes. The findings in this study are in line with the growing amount of scientific data suggesting an adverse effect of in-house exposure to wood smoke on respiratory health. Wood smoke is known to contain compounds such as carbon monoxide, nitrogen oxides, sulphur oxides, aldehydes, polycyclic aromatic hydrocarbons, and fine respirable particulate matter. All of these have been shown to cause deleterious physiologic responses in laboratory studies in humans.[32] Barregard et al.[20] demonstrated that wood smoke at levels that can be found in smoky indoor environments can cause an inflammatory response and signs of increased oxidative stress in the respiratory tract, especially in the lower airways. Exposure to wood smoke has been linked to an increased incidence of respiratory symptoms,[11] respiratory infections, low birth weight, cardiovascular events, and all-cause mortality both in adults and children,[6,12] and to lung cancer.[10]

Despite reports suggesting the potential role of NO as a sensitive biomarker for respiratory disease, no significant association between exhaled NO and wood smoke exposure was observed. Possibly, the changes in this healthy, young population were too limited to be detected using this biomarker. Moreover, exhaled NO appears to be very sensitive to acute inflammatory changes as observed during acute asthma, or when the maintenance dose of inhaled steroids is reduced, but levels tend to normalize in case of stabilized situations, such as stable asthma after steroid treatment.[17] The same rationale probably holds true for the lack of significant associations between wood fuel use and the lung function measurements or respiratory symptoms. Barregard et al.[20] reported increased levels of NO in study subjects at 3 hr after an acute exposure to wood smoke, but no differences were detectable anymore the next morning. The latter situation probably corresponds best to the situation of the adolescents in this study who were examined at school. As such, both studies did not yield contradictory results.

Despite being consistently observed, changes in the serum levels of CC16 (−6%) and of the CC16/SPD ratio (−9%) were relatively small compared to those

observed in diseased individuals or in subjects chronically exposed to tobacco smoke or toxic atmospheres in the industry.[18] These changes reveal thus the existence of early subclinical effects in the airways epithelium, which are most likely caused by irritating agents in wood smoke.[18,33,34] A decrease of CC16 and an increase of the CC16 over SPD ratio in serum can be explained by a decreased production of CC16, a major protein in the lung with anti-inflammatory activity[23] and the increased lung permeability. As such the observed biomarker changes might thus be indications of a subclinical process capable of shifting the respiratory system towards a status of increased vulnerability to pro-inflammatory processes.

The associations between wood smoke exposure and asthma, hay fever or sensitization against pollen as observed in this study are not in line with the information which could be retrieved in scientific literature. A large meta-analysis by Po et al.[12] did not detect an association between the exposure to biomass fuels and asthma in women and children, and little epidemiologic data were found linking wood smoke exposure to atopic outcomes. It is however striking that the association between sensitization and wood smoke exposure was rather specific. The positive association was very consistent for pollen, whereas it was not found with house dust mites. It is unlikely that it is a mere consequence of the fact that wood stoves are more frequently used in a rural area as we tried to address this by using both matching and multivariate approaches. We think that this specific association between wood fuel use and pollen sensitization might be causal and result from a significant in-house exposure to pollen carried over by wood.

We could not detect a statistically significant association between the CC16 to SPD ratio in serum and the risk of asthma, hay fever, and sensitization against pollen (Fig. 1), despite the fact that all were significantly associated with wood fuel use. Possibly, the increased frequencies of asthma, hay fever, and sensitization against pollen are not related to the cytotoxic/irritating activity of wood smoke which we consider as the most likely explanation for a causal link between wood fuel use and the altered CC16 to SPD ratio in serum. A modifying effect of wood smoke exposure nevertheless cannot be excluded since Samuelsen et al.[16] observed in an in vivo experiment that particles from wood smoke can act as adjuvant.

The allocation of a study participant to a sub-population with respect to wood fuel use was based on the only available source of information, that is, the interview inquiring about a heating system with wood at home. The lack of a more detailed characterization of exposure is the main limitation of this study. Because the sample collection took place in March, it can be reasonably assumed that the exposure to wood smoke occurred regularly during the preceding months. Moreover, despite the limitation related to the exposure characterization, it looks to us unlikely that the associations with wood fuel use which were consistently observed both after multivariate and matching approaches and which also involved objective measures, that is, biomarkers, resulted merely from confounding.

In conclusion, wood fuel use was associated with altered levels of biomarkers reflecting the integrity of the terminal airways epithelium. CC16 in serum was decreased, whereas SPD in serum tended to be increased, and, consequently, the CC16 to SPD ratio in serum was decreased. Wood fuel use was also associated with higher risks of asthma, hay fever, and sensitization against pollen allergens. Albeit that this study did not allow to elucidate the mechanisms underlying these associations, the results consistently pointed towards a possible involvement of wood fuel use in the development of these respiratory effects.

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Interactions between domestic water hardness, infant swimming and atopy in the development of childhood eczema

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ABSTRACT

Aim: Recent studies suggest that domestic water hardness and swimming in chlorinated pools may increase the prevalence of childhood eczema. The combined influence of these two factors as well as their interaction with atopic status has not been investigated.
Methods: We conducted a cross-sectional study on 358 children aged 5–6 years (54% of boys) in 30 kindergarten schools. Parents completed a questionnaire about the child's health, chlorinated pool attendance and potential confounders. Data about tap water quality were provided by water companies. Atopy was defined as a sensitization to at least one aeroallergen or as a medication for allergy. The effect of water hardness and infant swimming practice were assessed by multivariate logistic models. In addition, the effects of these risk factors combined with atopy were evaluated using two measures of biological interaction: the attributable proportion of interaction (AP) and the synergy index (S). AP > 0 and S > 1 indicate biological interaction between the two risk factors.
Results: Water hardness was linearly associated to the prevalence of eczema whereas the relationship of eczema with infant swimming was not linear. We observed a biological interaction between hard water (> 150 mg/L $CaCO_3$ L^{-1}) and atopic status that increases the prevalence of eczema with an odds ratio (OR) of 3.30 and a 95% confidence interval (CI) of 1.34–8.15 (AP, 0.41; 95% CI 0.15–0.66 and S, 2.4; 95% CI 0.96–6.01). Infant swimming practice combined with atopy also increased the prevalence of eczema (OR, 2.72; 95% CI 1.29–5.74) although none of the interaction measures was significant. However, when water hardness and infant swimming were combined, there was no further increase of the eczema prevalence due to some form of antagonistic interaction between these two factors (AP, −0.56; 95% CI −1.12 to −0.01 and S, 0.54; 95% CI 0.33–0.87).
Conclusions: Our study shows that exposure to hard water and infant swimming interact with atopic status to increase the prevalence of childhood eczema. A breaching of the epidermal barrier by detergents or salts in hard water and by chlorine-based oxidants in swimming pool water might explain these interactions.

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1. Introduction

Eczema is a chronic inflammatory disease characterized by alterations of the epidermal barrier function and an immunoglobulin E-mediated (IgE-mediated) sensitization to food and environmental allergens (Bieber, 2008). The disease develops as a result of complex interactions between various host (gender, family predisposition) and environmental factors (allergens, irritants and infectious agents). In these processes, skin barrier dysfunction appears as one of the most important components of eczema not only as a consequence of the skin inflammatory response but probably also as a factor promoting allergic sensitization and the resulting inflammatory response. The IgE-mediated sensitization often occurs several weeks or months after the first skin lesions appear, suggesting that the skin is the primary site of sensitization (Spergel and Paller Amy, 2003). The disease usually begins during infancy and frequently represents the first step of the atopic march that leads to the development of allergic rhinitis and asthma (Leung et al., 2004). The prevalence of eczema, like that of other atopic disorders, has steadily increased in industrialized countries over the last decades. Current estimates indicate that 10% to 20% of children in developed countries suffer from this condition (Williams et al., 2008). Although important, genetic factors cannot explain such a rapid rise of eczema, which can arise only from changes in our environment or lifestyle.

The hygiene hypothesis postulates that the increase of allergic diseases in most developed countries is the consequence of the declining exposure to microbial agents, especially during early life (Sherriff et al., 2002). An alternate hypothesis, which has been suggested more recently, relates the prevalence of eczema to the

Abbreviations: OR, odds ratio; CI, confidence interval; IgE, Immunoglobulin E; AP, attributable proportion of interaction; S, synergy index

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skin barrier disruption caused by environmental stressors. Of these stressors, those linked to the quality of tap or recreational water might be particularly relevant because exposure concerns the population at large and culminates precisely during infancy or early childhood. An association between water hardness and eczema prevalence has been observed in different ecological studies among schoolchildren (Arnedo-Pena et al., 2007; McNally et al., 1998; Miyake et al., 2004). Another potential causative factor linked to water is the exposure to chlorine-based irritants when attending swimming pools. Skin diseases, including eczema, are indeed more frequent in swimmers and workers exposed to swimming water (Lazarov et al., 2005; Basler et al., 2000; Font-Ribera et al., 2009; Bernard, 2007). All these studies, however, were focused on the effects of water hardness or swimming pool water taken separately and have not considered the possible interactions between these two factors, nor between these factors and the atopic status, which is the major driver of eczema risk.

We report here the results of a cross-sectional study focused on school-children in which we investigated the separate and combined effects of exposure to hard water, chlorinated pool attendance and atopy on the prevalence of eczema during childhood.

2. Material and methods

2.1. Study population

The study was conducted in 2008 on children recruited in 30 kindergarten located mainly in the areas of Brussels and Liege in Belgium. These schools belonged to two school networks who agreed to participate in the project: the school network of the municipality of Auderghem and the education network of the Liège area. All parents having children in third kindergarten in the schools of these two networks were invited to participate. A questionnaire and an informed consent document were distributed to a total of 839 children. The overall median participation rate was 51.3% and varied largely between schools (range 20–90%). Since there was no correlation between the participation rate and the prevalence of eczema, we decided to retain all schools for the statistical analysis and to include the participation rate among the potential predictors. The study protocol described in details previously (Voisin et al., 2010) was approved by the Ethics Committee of the Faculty of Medicine of Catholic University of Louvain.

Parental questionnaires were used to gather information about care during early life and all lifestyle or environmental factors susceptible to influence atopic diseases. The questionnaire included specific questions asking whether the child had ever been diagnosed by a doctor for most common childhood diseases, including eczema. For each question, the parents were asked to reply "yes" or "no". In the case of a positive reply, parents were asked to indicate the exact age at which the disease (thus eczema) was diagnosed. There were also questions about allergy medication ("Is your child under allergy medication?", "What is the name of the medication?"). The questionnaire also comprised specific questions intended to estimate the total time spent in outdoor or indoor chlorinated pools before the age of two years.

Data about water quality for years 2003 and 2007 were provided by the Public Department of Wallonia, the Vivaqua Company and the Brussels Institute for Management of the Environment. A value of water hardness was allocated to each child according to the postcode of residence of the 63 municipalities studied. Since data on water quality provided for the year 2007 were more completed, we used these data after having checked the good correlation with data of 2003 (Spearman's correlation test, $r=0.91$). When one municipality was supplied with distinct sources of water, the value for water hardness was estimated as the mean of the yearly averages of these different sources. Water hardness was expressed as mg of $CaCO_3\ L^{-1}$. The free chlorine concentration in tap water from all the municipalities was below 0.2 mg/L, which is the standard for tap water in Belgium.

Because the examination of children in schools precluded any provocation or blood test, screening for allergies was performed non-invasively by measuring specific IgE in nasal mucosa with the Rhinostick test. In comparison with the skin prick test, this test offers a similar sensitivity and a greater specificity (Marcucci et al., 2004). Among children for who water hardness data were available, the test was successfully performed in 358 children. The following allergens were screened: cat epithelium, *Dermatophagoides pteronyssinus*, *Anthoxanthum odoratum*, *Parietaria officinalis* and a mix of four allergens containing *Betula odorosa*, *Corylus avellana*, *Carpinus betulus* and *Alnus incona*. Children were considered as atopic when they were sensitized to at least one of the above allergens (specific IgE >0.35 kIU/l). Children with a negative Rhinostick test but under medication for allergy, as specified in the questionnaire, were also classified as atopics.

2.2. Statistical analysis

Multiple logistic regression analysis was used to assess the adjusted odds ratio (OR) and their 95% confidence intervals (CI) of eczema associated with water hardness and chlorinated pool attendance during infancy. Age, gender, parental eczema and/or allergy, breastfeeding, parent's education level (high, minimum one parent in possession of a bachelor educational level or higher), presence of older siblings, passive smoking, maternal smoking during pregnancy, participation rate (above 50% or not) were selected as *a priori* potential confounding factors. To study these associations, we first assessed whether or not water hardness and infant swimming practice were linearly related to the log-odds of eczema using a restricted cubic spline model (Software R 2.12.1). The null hypothesis that the log-odds of eczema was a linear function was accepted only for the water hardness but rejected for infant swimming. Water hardness was thus tested as a continuous variable. However, to exploit the wide range of water hardness of our study with values up to 420 mg/L, which is quite uncommon (WHO, 1996), we also analyzed this variable stratified in three categories of increasing values (soft: <150 mg/L $CaCO_3\ L^{-1}$, moderately hard: 150–350 mg/L $CaCO_3\ L^{-1}$and very hard: >350 mg/L $CaCO_3\ L^{-1}$). These cut-off points were selected because they corresponded to the standard water hardness categories used in Belgium. Of interest, these limits also corresponded to the 25th and 50th percentiles of water hardness distribution of our database. To examine the nonlinear relationship between infant swimming and prevalence of eczema, we modeled infant swimming by restricted cubic spline with four knots at percentiles 5%, 35%, 65%, and 95% in a logistic regression. Unadjusted trends for increasing eczema prevalence with increasing water hardness were assessed with the Cochran–Armitage trend test.

We tested potential statistical and biological interactions between atopy, water hardness and infant swimming practice on the prevalence of eczema. Statistical interactions were examined by including the appropriate product interaction term in the multivariate logistic regression. Biological interactions in the regression models were tested as departure from additivity. We use the two indices proposed by Rothman and Greenland (1998) to measure these interactions from logarithmic models: the attributable proportion of interaction (AP) and the synergy index (S). The AP estimates the attributable proportion of eczema, which is due to the interaction in children with both exposures. The S examines whether the proportion of children with eczema is significantly different from the one obtained among children exposed to the additive effect of the two risk factors taken separately. First, atopy, infant swimming and water hardness were combined two by two in three new variables with four levels summarized as follows: a level of joint exposure to both risk factors (A^+B^+), a level of exposure to one of the risk factors only (A^+B^- or A^-B^+) and a level of reference of no exposure (A^-B^-). On the basis of adjusted odds ratios obtained in the logistic regression models, we calculated the two measures of interaction on an additive scale and their corresponding confidence intervals by using an Excel spreadsheet set up by Andersson et al. (2005). There is a biological interaction when AP (95% CI) does not include 0 and S (95% CI) does not cross 1.

3. Results

The study population included 358 schoolchildren (54% of boys) with a mean age of 5.7 years (standard deviation, 0.4). Main characteristics of these children are summarized in Table 1 for the whole population and separately for children who had a doctor-diagnosed eczema ($n=94$, 26%) and those who had not. Table 1 also shows the odds ratios and their 95% confidence interval for eczema associated with the described characteristics. A significant increase of eczema prevalence was associated to atopic status, parental allergies or eczema, maternal education, domestic water hardness and cumulative pool attendance before the age 2 years. There were no significant differences between the two groups in the proportions of children sensitized to allergens, children who had been breastfed or those who had been exposed to older siblings or tobacco smoke. Although the hardness of tap water was on average higher in children with reported eczema, the proportions of children having access to hard water (>150 mg/L $CaCO_3\ L^{-1}$) did not differ between the two groups. Proportion of infant swimmers was higher in the eczema group but the hours spent in chlorinated pool by these infant swimmers were not higher. There was no association between the prevalence of atopy and the exposure to hard water or infant swimming practice. Last, the proportion of infant swimmers was lower in the soft water group than in the hard water group (31% vs 51%,), meaning that the specific influence of hard water and infant

swimming can be accurately assessed only by the mutual exclusion of these factors.

Table 2 shows the crude and adjusted odds ratio of eczema associated with water hardness and infant swimming in the whole population and separately for non-atopic and atopic children. When tested as a continuous variable, water hardness was associated with an increased eczema prevalence independently of the atopic status. However, when testing water hardness as a categorical variable, the odds for eczema was significantly increased only among atopic children exposed to very hard water (>350 mg/L $CaCO_3$ L^{-1}, OR, 3.36; 95% CI 1.02–11.1). The adjusted odds ratio for eczema associated to the upper *quartile* of infant swimming compared with lower was 1.41 (95% CI 0.98–2.04) in all children and was statistically significant among non atopic children (OR, 1.78; 95% CI 1.09–2.89).

In order to assess the specific influence of water hardness and infant swimming, we repeated the logistic analyses by mutually excluding each of these factors. The results shown in Table 3 revealed that these two factors were mutually confounding their associations with prevalence of eczema. Non-infant swimmers showed indeed a strong association between water hardness and eczema prevalence (OR, 3.76; 95% CI 1.31–10.76), which completely disappeared in their peers who had swum during infancy (OR, 0.76; 95% CI, 0.27–2.14). Similarly, in children using soft water, infant swimming and prevalence of eczema showed a tendency to be associated (OR, 2.13; 95% CI 0.98–4.64), which was not seen in those having access to moderately or very hard water (OR, 1.26; 95% CI 0.79–2.02).

Given the above influence of water hardness, infant swimming and atopy on the prevalence of eczema, we further assess the possible interactions between these three factors. When introducing the interactive terms in the logistic regression models, there was no evidence of statistical interaction (OR (atopy *x* hard water), 1.38; 95% CI 0.43–4.36, OR (atopy *x* infant swimming), 0.86; 95% CI, 0.31–2.36, OR (infant swimming *x* hard water), 0.40; 95% CI 0.12–1.33). By contrast a series of biological interactions as a departure from additivity were found by calculating the AP and S interaction measures. Table 4 shows the odds ratios for each factor tested separately and in combination with each other. The interaction measures calculated on the basis of these odds showed a significant synergistic interaction between atopy and water hardness (AP, 0.41; 95% CI 0.15–0.66 and S, 2.40; 95% CI 0.96–6.01). Although the odds for eczema associated to infant swimming combined with atopy was higher than the odds for each factor alone, the additive interaction measures were not statistically significant (AP, 0.04; 95% CI -0.34 to 0.42 and S, 1.07; 95% CI 0.57 to 2.0). The same analysis revealed a significant antagonistic effect between hard water and infant swimming practice on eczema (AP, -0.56; 95% CI -1.12 to -0.01 and S, 0.54; 95% CI 0.33–0.87). Indeed, children with both exposures reported a prevalence of eczema that was lower than the additive effect of each risk factor.

4. Discussion

While confirming the increased prevalence of childhood eczema associated with exposure to hard water and chlorinated pools, our study shows that this increase in prevalence largely stems from their biological interactions with the atopic status. Both infant swimming and exposure to hard water were indeed significantly associated with prevalence of eczema only when combined with atopic status. Our study also shows that by interacting with each other, hard water exposure and infant swimming mutually weaken their association with eczema, resulting in a complex pattern of confounding effects. Clearly, the failure to consider all these interactive effects may lead to an underestimation of the prevalences of eczema associated with hard water or infant swimming.

Interactions reported in epidemiological studies should always be interpreted with caution. We are, however, rather confident that the interactions found in this study arise from the biological interactions between the exposure to skin irritating substances and the genetic

Table 1
Characteristics of study participants according to eczema and associations of covariates with the prevalence eczema.

	All ($n=358$)	Eczema		OR for eczema (95% CI) [b]
		No ($n=264$)	Yes ($n=94$)	
Age, mean (SD [a])	5.7 (0.4)	5.7 (0.4)	5.7 (0.4)	0.86 (0.47–1.59)
Boys, *n* (%)	294 (54.2)	139 (52.7)	55 (58.5)	1.27 (0.79–2.04)
Body mass index, mean (SD [a])	20.7 (3.2)	20.7 (3.2)	20.8 (3.2)	1.01 (0.94–1.09)
Mother and/or father with eczema, *n* (%)	63 (17.6)	38 (14.4)	25 (26.6)	2.16 (1.22–3.82)
Parents with allergies or eczema, *n* (%)	164 (45.8)	109 (41.3)	55 (58.5)	2.01 (1.24–3.23)
Older sibling ≥ 1, *n* (%)	203 (56.7)	154 (58.3)	49 (52.1)	0.78 (0.49–1.25)
Parental education level (high, bachelor level)				
Maternal education, *n* (%)	240 (67.0)	168 (63.6)	72 (76.6)	1.87 (1.09–3.21)
Paternal education, *n* (%)	218 (60.9)	160 (60.6)	58 (61.7)	1.05 (0.65–1.70)
Passive smoker at home, *n* (%)	114 (31.8)	90 (34.1)	24 (25.5)	0.66 (0.39–1.13)
Maternal smoking during pregnancy, *n* (%)	46 (12.8)	32 (12.1)	14 (14.9)	1.27 (0.64–2.50)
Breastfeeding, *n* (%)	279 (77.9)	206 (78.0)	73 (77.7)	0.98 (0.56–1.72)
Domestic water hardness,				
Median (IQR [a]), mg/L $CaCO_3$ L^{-1}	34.5 (9.1–36.2)	34.5 (9.1–36.2)	36.2 (19.3–36.5)	1.02 (1.00–1.04)
Water hardness >150 mg/L $CaCO_3$ L^{-1}, *n* (%)	265 (74.0)	190 (72.0)	75 (79.8)	1.54 (0.87–2.72)
Cumulative pool attendance before 2 years,				
Median (IQR [a]), hours	24.0 (10.8–59.0)	23.9 (8.8–48.4)	27.2 (13.0–69.0)	1.00 (1.00–1.01)
Infant swimmers, *n* (%)	164 (45.8)	116 (42.4)	52 (55.3)	1.68 (1.05–2.70)
Atopic status,				
At least 1 aeroallergen >0.35 kIU/L, *n* (%)	86 (24.0)	60 (22.7)	26 (27.7)	1.30 (0.76–2.22)
Current Medication for allergy, *n* (%)	59 (16.5)	34 (12.9)	25 (26.6)	2.45 (1.37–4.39)
Sensitization to at least 1 aeroallergen or medication for allergy, *n* (%)	121 (33.8)	80 (30.3)	41 (43.6)	1.78 (1.10–2.89)
Doctor-diagnosed eczema, *n* (%)	94 (26.3)	/	/	/

[a] IQR: interquartile range; SD: standard deviation.
[b] OR, odds ratio; 95% CI, 95% confidence interval.

Table 2
Prevalences and odds ratio for eczema associated with water hardness and infant swimming practice according to atopic status.

	All children				Non atopic				Atopic			
	N	(%)	Crude OR (95% CI) [a]	Adjusted OR (95% IC) [b]	*N*	(%)	Crude OR (95% CI) [a]	Adjusted OR (95% IC)[b]	*N*	(%)	Crude OR (95% CI) [a]	Adjusted OR (95% IC) [b]
Water hardness (mg/L $CaCO_3$ L^{-1})												
continuous	358	26.3	1.02 (1.00 –1.04)	1.02 (1.00–1.05)	237	22.4	1.02 (0.99–1.05)	1.03 (1.00–1.06)	121	33.9	1.03 (1.00–1.06)	1.04 (1.00–1.07)
< 150	93	20.4	1.00	1.00	55	18.2	1.00	1.00	38	23.7	1.00	1.00
150–350	96	25.0	1.30 (0.66 –2.57)	1.39 (0.67–2.88)	65	21.5	1.24 (0.5–3.05)	1.15 (0.44–3.02)	31	32.3	1.53 (0.53–4.44)	2.05 (0.63–6.65)
> 350	169	30.2	1.68 (0.92 –3.07)	1.97 (0.97–3.96)	117	24.8	1.48 (0.66–3.31)	1.77 (0.70–4.43)	52	42.3	2.36 (0.93–5.98)	3.36 (1.02–11.1)
P for trend	–	0.08	–	–	0.32	–	–	0.06	–	–	–	–
Infant swimming												
RCS [c]	358	26.3	1.51 (1.06–2.16)	1.41 (0.98–2.04)	237	22.4	1.96 (1.24–3.10)	1.78 (1.09–2.89)	121	33.9	1.05 (0.50–2.18)	0.94 (0.42–2.10)

[a] OR, Odds ratio; 95% CI, 95% confidence interval.
[b] Adjustment for age, gender, parental eczema and/or allergy, breastfeeding, parent's education level, presence of older siblings, passive smoking, maternal smoking during pregnancy and participation rate.
[c] The OR were estimated for the upper quartile by using restricted cubic spline (RCS) with the lowest quartile as the reference group.

Table 3
Prevalences and odds ratios for eczema associated with water hardness and infant swimming practice after stratification for these two risk factors.

Water hardness (mg/L $CaCO_3$ L^{-1})	Non infant swimmers				Infant swimmers			
	N	(%)	Crude OR (95% IC)[a]	Adjusted OR (95% IC)[b]	*N*	(%)	Crude OR (95% IC) [a]	Adjusted OR (95% IC)[b]
continuous	194	21.6	1.02 (1.00–1.05)	1.04 (1.01–1.08)	164	31.7	1.02 (0.99–1.05)	1.03 (1.00–1.06)
< 150	64	14.1	1.00	1.00	29	34.5	1.00	1.00
150–350	51	27.5	2.31 (0.91–5.89)	3.62 (1.26–10.4)	45	22.2	0.53 (0.19–1.54)	0.45 (0.15–1.38)
> 350	79	24.1	1.94 (0.81–4.64)	3.76 (1.31–10.76)	90	35.6	1.05 (0.44–2.53)	0.76 (0.27–2.14)
P for trend			0.17				0.56	
Infant swimming	Soft water (< 150 mg/L $CaCO_3$ L^{-1})				Hard water (> 150 mg/L $CaCO_3$ L^{-1})			
	N	%	Crude OR (95% IC)[a]	Adjusted OR (95% IC)[b]	*N*	Prevalence	Crude OR (95% IC)[a]	Adjusted OR (95% IC)[b]
RCS [c]	93	20.4	2.01 (0.98–4.09)	2.13 (0.98–4.64)	265	28.3%	1.40 (0.90–2.18)	1.26 (0.79–2.02)

[a] OR, Odds ratio; 95% CI, 95% confidence interval.
[b] Adjustment for age, gender, parental eczema and/or allergy, breastfeeding, parent's education level, presence of older siblings, passive smoking, maternal smoking during pregnancy and participation rate.
[c] The OR were estimated for the upper quartile by using restricted cubic spline (RCS) with the lowest quartile as the reference group.

Table 4
Biological interactions between atopy, water hardness and infant swimming.

Interaction categories	*N*	Prevalence (%)	Crude OR (95% CI)[a]	Adjusted OR (95% IC)[b]	AP (95% IC)[a]	S (95% IC)[a]
Interaction atopy + hard water [c]						
No atopy + soft water	55	18.2	1.00	1.00	0.41	2.40
Atopy	38	23.7	1.40 (0.51–3.85)	1.37 (0.48–3.92)	(0.15 to 0.66)	(0.96 to 6.01)
Hard water	182	23.6	1.39 (0.65–2.99)	1.59 (0.69–3.64)	–	–
Atopy + hard water	83	38.6	2.82 (1.25–6.38)	3.30 (1.34–8.15)	–	–
Interaction atopy + infant swimming						
No atopy + no infant swimming	126	17.5	1.00	1.00	0.04	1.07
Atopy	68	29.4	1.97 (0.98–3.95)	1.89 (0.93–3.86)	(−0.34 to 0.42)	(0.57 to 2.00)
Infant swimming	111	27.9	1.83 (0.99–3.40)	1.67 (0.88–3.19)	–	–
Atopy + infant swimming	53	39.6	3.10 (1.51–6.36)	2.72 (1.29–5.74)	–	–
Interaction hard water + infant swimming [c]						
Soft water + no infant swimming	64	14.1	1.00	1.00	−0.56	0.54
Hard water	130	25.4	2.08 (0.93–4.66)	2.36 (0.98–5.68)	(−1.12 to −0.01)	(0.33 to 0.87)
Infant swimming	29	34.5	3.22(1.14–9.12)	2.95 (1.01–8.58)	–	–
Hard water + infant swimming	135	31.1	2.76 (1.25–6.10)	2.82 (1.19–6.70)	–	

[a] OR, Odds ratio; 95% CI, 95% confidence interval; AP, attributable proportion of interaction; S, synergy index.
[b] Adjustment for age, parental education level, parental allergy and/or eczema, maternal smoking during pregnancy, presence of older siblings, gender, breastfeeding, participation rate and passive smoker.
[c] Soft water < 150 mg/L $CaCO_3$ L^{-1}; hard water > 150 mg/L $CaCO_3$ L^{-1}.

predisposition to atopic diseases. Although it is known since long that hard water can irritate the skin, the involved mechanisms are still unclear. The explanations commonly forwarded are the deposition of calcium salts on the skin and the use of larger amounts soap and shampooing to lather the water (Warren et al., 1996; White et al., 1987; Ananthapadmanabhan et al., 2004). Chlorine used to disinfect

swimming pool water and its by-products such as chloramines are potent oxidants that can also cause skin irritation (Lazarov et al., 2005; Basler et al., 2000; Font-Ribera et al., 2009; Bernard, 2007). For instance, Seki et al. (2003) reported that the water holding capacity of the *stratum corneum* decreased after immersion in chlorinated water. Gardinier et al. (2009) have shown that a single swimming session leads to a substantial removal of the sebum from the skin surface. This degreasing action of pool water increases skin pH from acidic to neutral pH, which in turn can alter the skin homeostasis and permeability (Mauro et al., 1998). Furthermore, water *per se* is a well-known skin irritant that can disrupt skin barriers as revealed by an increased transepidermal water loss (Ramsing and Agner, 1997; Tsai and Maibach, 1999). All these chemical stressors might quite conceivably increase the permeability of skin epithelial barriers and thereby facilitate the penetration of allergens and the development of eczema (Löffler et al., 2003; Boralevi et al., 2008; Mattila et al., 2011). This hyperpermeability mechanism triggered by skin irritation would be basically the same as that proposed for the increased risks of asthma and respiratory allergies observed among atopic swimmers regularly attending chlorinated swimming pools (Bernard, 2007).

Our study suggests that atopic subjects might benefit from washing or bathing with soft water or from swimming in non-chlorinated pools. At this stage, however, it is unknown whether avoidance of irritants in tap or swimming water could be beneficial for preventing childhood eczema or for alleviating eczema skin symptoms. With regard to hard water, a recent trial testing the effect of water softeners on eczema severity in children, showed no additional benefit after the installation of an ion-exchange water softener (Thomas et al., 2011). If these findings are confirmed, this would suggest that the association between eczema and water hardness reported by us and other studies (Arnedo-Pena et al., 2007; McNally et al., 1998; Miyake et al., 2004) will be related more to the initiation of eczema and in particular to the early steps of the skin sensitization. The same reasoning could be held for pool chlorine, which might also act more as an adjuvant of skin sensitization than as an irritant exacerbating the symptoms of eczema.

Our study presents several limitations. The lack of a detailed characterization of the cumulative exposure to hard water and swimming pool water is the main limitation of this study. For the exposure to hard water, we had a good measure of the hardness of tap water supplied to each participant but we had no estimate of the contact time while for infant swimming we had just the opposite information. It looks to us unlikely that this lack of information could have distorted our analyses. Skin contact with tap water during infancy is indeed mainly related to washing or bathing, i.e., activities that should not greatly vary between infants. As to the irritating potential of chlorinated pools, it is probably mainly determined by the concentrations of active and combined chlorine, which in public pools at least, are maintained within a narrow range of concentrations to comply with the legislation. Moreover, our data on time spent in chlorinated pool might be subjected to recall bias and could lead to a potential exposure misclassification bias. The association, however, also emerges when considering ever swimming, for which memory bias is less likely. Another limitation is the relatively low participation rate, which was almost unavoidable given the very young age of participants and the fact that our study required the application of medical tests in schools. The participation, however, was unrelated to the outcome and the main risk factors, making participation biases rather improbable.

In conclusion, our study shows that exposure to hard water and, to a lesser extent, infant swimming practice, interact with atopy to increase the prevalence of childhood eczema. A breaching of the epidermal barrier by detergents or salts in hard water and by chlorine-based oxidants in swimming pool might explain these interactions.

Conflict of interest

None of the authors has a conflict of interest to disclose.

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Risks of new-onset allergic sensitization and airway inflammation after early age swimming in chlorinated pools

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ABSTRACT

Rationale: Irritant chlorination products in swimming pools can cause respiratory problems in swimmers but their possible implication in allergies development is still unclear.
Objectives: To assess prospectively whether early-life attendance at chlorinated pools increases the risks of IgE sensitization and of airways inflammation later during childhood.
Methods: We conducted a two-year prospective study among 196 kindergarten children (mean age of 5.7 years, 54% of boys). We measured exhaled nitric oxide (eNO) and aeroallergen-specific IgE in nasal mucosa. Parents completed a questionnaire about the child's health, chlorinated pool attendance and potential confounders.
Main Results: Ever swimming at indoor or outdoor chlorinated pools before the age of three years was associated with higher odds for new-onset IgE sensitization to house dust mite (adjusted odds ratio [aOR] 2.93, 95% confidence interval [CI] 1.14–7.55) and for new-onset increased eNO (>15 ppb; aOR, 4.54, 95% CI 1.48–13.9). For both outcomes, aORs increased dose-dependently with time spent in chlorinated pools with values reaching, respectively, 3.60 (95% CI 1.21–10.7) and 5.92 (95% CI 1.72–20.5) when the cumulative pool attendance exceeded 60 h These risks appeared independently of each other, of parental history of allergies and of pre-existing diseases, including eczema, which at baseline was more prevalent in early swimmers (aOR, 2.91; 95% CI 1.23–6.89). Such associations were not seen with IgE sensitization to pollen or cat allergens.
Conclusion: Attendance at chlorinated swimming pools in early life is associated with higher risks of new-onset airways inflammation and IgE sensitization to house dust mite, independently of other risk factors.

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Introduction

Atopic eczema, rhinitis and asthma are the most prevalent chronic diseases in children of the developed world (Mallol et al., 2012). These diseases have usually their onset during early life and then they develop sequentially along a pathway called the allergic or atopic march (Ker and Hartert, 2009; Spergel, 2010; Zheng et al., 2011). Although the links between these different types of allergic disease are not fully understood, the prevailing paradigm assumes that the atopic march starts with atopic dermatitis, then proceeds to allergic rhinitis to end with asthma (Martin et al., 2011).

Factors driving the atopic march and the epidemic of allergies are largely unknown. Current hypotheses postulate that these factors are related to the hygiene practices of the Western world. According to the classical hygiene hypothesis (Strachan, 1989), the increase of allergic diseases in developed countries would be the consequence of the reduced exposure to certain microbial agents during early life when the immune system is maturating (Strachan, 1989, 2000). The hygiene hypothesis postulates that an insufficient stimulation of the immune system early in life shifts the immune system from a Th1- to a Th2-cell response, which leads to IgE-mediated diseases such as eczema, asthma and hay fever (McGeady, 2004). Some recent studies, however, give reasons for questioning the validity of the hygiene hypothesis as an unifying explanation for the worldwide rise of allergies. Among these, there is the emerging concept that allergic diseases are primarily epithelial disorders driven by airway barrier dysfunction caused by environmental insults (Holgate et al., 1999; Cookson, 2004). Also challenging the hygiene hypothesis, recent studies have failed to confirm the protective role of infectious exposures, older siblings or Bacillus Calmette-Guèrin (BCG) vaccination toward the risk of allergic outcomes (Cramer et al., 2012; Flohr et al., 2012; Brunekreef et al., 2012).

Another hypothesis closely linked to hygiene is the "chlorine or chlorination hypothesis". This hypothesis proposes that the rise of allergic diseases in Westernized countries is, at least partly, driven

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by the increasing and largely uncontrolled exposure of children to chlorination products, especially in swimming pools (Bernard et al., 2003; Bernard, 2007). Because of their strong oxidizing potential, chlorine and its derivatives such as chloramines can open tight junctions of epithelial barriers and thereby promote the allergen delivery to dendritic cells and the mounting of the Th2 immune response. Several studies have indeed shown that chlorinated pool attendance is associated with increased risks of allergic diseases including hay fever, allergic rhinitis and asthma (Bernard, 2007; Bernard et al., 2006; Kohlhammer et al., 2006; Bernard et al., 2009; Cotter and Ryan, 2009; Ferrari et al., 2011). These risks, which increase dose-dependently with the cumulative pool attendance (CPA), appear to largely stem from interactions with the atopic status (Bernard et al., 2006, 2009). To be triggered, these pool chlorine/atopy interactions require thus a pre-sensitization to aeroallergens as well as a cumulative exposure to chlorinated pools lasting at least a few years. These two conditions, with perhaps some misclassification biases (Bernard et al., 2011; Klootwijk and Krul, 2011), might explain the apparent inconsistencies between studies in young children (Schoefer et al., 2008; Font-Ribera et al., 2009, 2011) and those conducted in adolescents or adults (Bernard et al., 2009; Cotter and Ryan, 2009; Ferrari et al., 2011).

The influence of chlorinated pools on the development of allergic diseases might, however, not be limited to interactions promoting the clinical manifestations of atopy (Bernard et al., 2011). Some observations suggest that chlorinated pool attendance might also increase the risks of allergen sensitization and therefore contribute to the development of the atopic status. The attendance at indoor or outdoor chlorinated pools during infancy or early childhood has been associated with an increased risk of house dust mite (HDM) sensitization (Bernard et al., 2008; Jacobs et al., 2012), which is one of strongest predictors of childhood rhinitis and asthma (Lodge et al., 2011). Studies among infant swimmers have also shown that this practice is associated with an increased risk of eczema, one of the earliest signs of the atopic march (Font-Ribera et al., 2009; Chaumont et al., 2012).

As all these findings were based on cross-sectional studies with a retrospective assessment of swimming pool attendance, the possibility of recall bias, exposure misclassification or even of reverse causation cannot be formally excluded. The aim of this prospective study was thus to further assess whether attendance at chlorinated pools early in life may increase the risks of aeroallergen sensitization as well as of increased exhaled nitric oxide (eNO), a predictor also of rhinitis and asthma in children (Olin et al., 2010; Malinovschi et al., 2012).

Materials and methods

Study population

This study was conducted in the frame of a larger study intended to assess the impact of air pollution on the respiratory health of children (Voisin et al., 2010). We aimed to recruit all children in third kindergarten in 30 schools located in the areas of Brussels and Liège in Belgium. An informed consent document and a questionnaire were provided to the parents. Of the 839 children who received these documents, 430 returned the questionnaire and the consent document signed by the parents. These children were examined in schools between December 2007 and March 2008. Two years later, between December 2009 and March 2010, we visited the schools in the same order to re-examine these children. Of the initial baseline population, 128 children had left the schools, which left for the follow-up a population of 302 children. Among these, 236 (78.1%) participated to the study and of them 196 could perform successfully all the tests. Participants and non-participants did not differ significantly regarding studied outcomes, chlorinated pool attendance and the main risk factors of allergies. All children were examined only with the written informed consent of their parents. In addition, just before performing the tests in schools, we verbally assured to have the assent of each child. The ethics committee of the Faculty of Medicine of the Catholic University of Louvain approved the study protocol, which complied with applicable requirements of the international regulations.

Questionnaire

Parents completed a self-administered questionnaire addressing aspects related to social and medical characteristics of the child and its family, the in- and out-house environment and recreational activities including swimming. For swimming practice, parents were asked to specify the type of swimming pool attended by their child, the type of disinfection method used (some children had access to copper-silver sanitized pools), the frequency of attendance and the age at which their child started to attend the pool on a regular basis. This information served to calculate the cumulative attendance of indoor and/or outdoor chlorinated pools, before the age of 3 years (what was referred to as early swimming), during the two-years follow-up and over lifetime, at the mean age of 5.7 or 7.5 years. The questionnaire also included questions asking whether the child had ever been diagnosed for most common respiratory or allergic diseases, including bronchiolitis, eczema, asthma, hay fever and allergic rhinitis.

Exposure to chlorination and its by-products

In Belgium, public swimming pools (all indoor in the studied areas) are legally required to monitor the microbial and chemical quality of the water by maintaining several parameters within regulatory limits, including free chlorine (0.5–1.5 ppm) and combined chlorine (<0.8 ppm) in water and trichloramine in pool air (<500 $\mu g/m^3$ in air sampled 1.5 m above the pool surface). There are no specific regulations for privately owned swimming pools, which are disinfected according to the instructions of the chlorine supplier (recommended free chlorine, 1–2 ppm). An additional potential source of exposure to chlorination products for our children was via the use of tap water, in particularly for showering or bathing. Data about chlorine levels in tap water for the years 2003 were provided by the Public Department of Wallonia, the Vivaqua Company and the Brussels Institute for Management of the Environment. Data were allocated to each child according to the postcode of residence of the 63 municipalities studied. The mean concentration of free chlorine in tap water (regulatory limit, 200 $\mu g/l$) of our study participants was 65 $\mu g/l$, a value more than 10 times lower than levels in swimming pools. The concentration of THM, the major chlorination by-product in tap water, did not exceed 10 $\mu g/l$.

Samples collection and analyses

Children were examined in their schools between 9:00 and 13:00. The protocol comprised the measurement of height and body weight, a screening of aeroallergen sensitization using the Rhinostick test, a non-invasive test having 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 $\geq$ 0.35 kIU/l (Marcucci and Sensi, 1989). The concentration of nitric oxide (NO) was measured in exhaled air with the NIOX^{TM} analyzer (Aerocrine AB, Solna, Sweden) by following the guidelines of the American Thoracic Society (1999). For diagnosing increased eNO values, we used a cut-off of 15 ppb

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(Buchvald et al., 2005), which in our study corresponded to the 95th percentile of baseline values in non-atopic children.

Statistical analyses

Results were reported as mean ± SD or as median with interquartile range for non-normally distributed variables. Differences between early swimmers and controls were assessed using the Chi-squared test for binary variables and the Student's *t*-test or the Mann–Whitney *U*-test for continuous variables. Associations between early swimming and outcomes were assessed by logistic regression analyses. We adopted a backward approach that consisted to include all potential control variables and to remove the least significant predictor until the model only contained variables with a *p*-value of <0.20. We tested 17 potential predictors including age, gender, maternal smoking during pregnancy, preterm birth, delivery by cesarean, breastfeeding, day care attendance, number of older siblings, probiotics, rural versus urban area, vicinity to a busy road (<100 m), parental asthma, parental allergies, high parental educational level (father and/or mother graduate of university or high school), parental smoking, house cleaning with bleach, double-glazed windows, mold on bedroom walls, air fresheners and chlorinated pool attendance (all types of pools combined). We analyzed exposure-response relationships by stratifying children in categories of increasing cumulative pool attendance (CPA). For the attendance at chlorinated pools before the age of three (referred to early swimming), we used as referents children who never attended chlorinated pools before the age of 3 and we dichotomized early swimmers at CPA cut-off values (30 or 60 h) that corresponded approximately to the median (32 h) or twice the median of their CPA. We also selected these two cut-off values because they were respectively equivalent to a 30 min swimming session fortnightly or weekly during three first years of life. As there were only 10 children who never visited chlorinated pools, the CPA during the follow-up or over lifetime was stratified in tertiles of increasing CPA. 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

Table 1 compares the characteristics of children who had attended chlorinated pools before the age of three with their peers who did not, both at baseline and at the end of the two-year follow up period. Early swimmers had on average spent a total of 33 h in indoor or outdoor chlorinated pools, which over a period of three years corresponds approximately to a 30 min swimming session fortnightly. These early swimmers continue to swim more frequently than their peers later so that their CPA during the follow-up and over lifetime was more than twice that of controls. Except for chlorinated pool exposure, early swimmers and their controls were well matched for gender, parental history of atopic diseases and the exposure to indoor or outdoor pollutants. Proportions of children who had been breastfed or had attended a day care center were however higher among early swimmers than in their peers.

At baseline, early swimmers and controls were well matched for sensitization to aeroallergens, the two groups having almost the same prevalences of sensitization to HDM (13.6 vs. 14.3%) and pollen (17.9 vs. 16.1%). There prevalence of elevated eNO in early swimmers (12.1%) was almost twice lower than that of controls (23.2%) but after adjustment for other covariates this difference was not statistically significant. The two groups also did not show any meaningful difference in the baseline prevalences of doctor-diagnosed respiratory or allergic diseases except for eczema which much more frequent in early swimmers (aOR 2.91, 95% CI 1.23–6.89, $p = 0.02$).

Table 3 shows the prevalences of studied outcomes at the end of the two-year follow-up period. Compared to controls, early swimmers were significantly more likely to develop new-onset HDM sensitization (aOR, 2.93, 95% CI 1.14–7.55, $p = 0.03$) and increased eNO (aOR, 4.54, 95% CI 1.48–13.9, $p = 0.008$). These associations were largely independent of each other, being moderately affected by the mutual exclusion of the two outcomes (aOR for new-onset HDM without increased eNO; 2.95, 0.96–9.02, $p = 0.06$; aOR for new-onset increased eNO with no HDM sensitization, 2.96, 0.93–9.44, $p = 0.07$). Excluding children with a history of eczema at baseline who were overrepresented among early swimmers did not weaken either these associations between early swimming and new-onset HDM sensitization (aOR, 3.27, 95% CI 1.04–10.3, $p = 0.02$) or increased eNO (aOR, 3.52, 95% CI 1.07–11.5, $p = 0.04$). Nor were these associations weakened by the exclusion of bronchiolitis, asthma and other pre-existing diseases. There was a trend toward a lower prevalence of new-onset sensitization to pollen among early swimmers but this trend was largely abolished by the exclusion of cases of eczema at baseline (aOR, 0.47, 95% CI 0.12–1.77, $p = 0.26$). Because of the pre-existing differences at baseline, this pattern of changes did not really emerge when considering all cases at the end of follow-up. The increase in the prevalence of HDM sensitization in early swimmers was of borderline significance ($p = 0.056$) and the increase in eNO completely lost its statistical significance. Whether considering all or new-onset cases, there were no significant differences between early swimmers and controls in the prevalences of doctor-diagnosed allergic rhinitis, hay fever and asthma at the end of the follow-up.

Because remission of allergic sensitization is rather frequent during early childhood, especially in case of mono-sensitization (Matricardi et al., 2009), we examined whether chlorinated pool attendance could influence this phenomenon as well as the possible remission of increased eNO and other outcomes. During the follow-up, sensitization to HDM and to pollen resolved in 15 and 26 children, respectively, and there were 12 cases of remission for increased eNO. Remissions rates for these outcomes were not influenced by the time spent in chlorinated pools during the follow-up (all p for trend>0.20) and they did not differ significantly between early swimmers and their controls (57.9 vs 50%; 76.0 vs 77.8% and 38.5% vs 41%, respectively, all $p > 0.50$). No associations were seen also between swimming in chlorinated pools and the remission of other outcomes (results not shown).

We pursued our analyses by examining the exposure-response relationships between new-onset cases of allergic sensitization or increased eNO and the early swimming practice using as exposure metric the number of hours children had spent in chlorinated pools before the age of three years. We categorized early swimmers in low versus high or low versus very high CPA using as cutoff points the values of 30 h or 60 h, which approximated respectively the median (32 h) or twice the median of the CPA values of early swimmers before the age of three years. Table 4 shows that for both types of stratification, the adjusted prevalences of new-onset HDM sensitization and elevated eNO increased dose-dependently with the cumulative exposure to chlorinated pools during early life. There were yet some differences between the two outcomes in that the exposure-response relationship emerged at higher CPA for elevated NO than for HDM sensitization. Quite remarkably, all these associations were strengthened by the exclusion of children with reported eczema at baseline or with a parental history of allergic diseases. The same analyses done with new-onset pollen sensitization, which tended to be lower in early swimmers – Table 2, did not reveal any relationship with the time spent of chlorinated pools before three the age of three years (p for trend = 0.54 and 0.77 for stratification at a CPA of 30 h or 60 h, respectively). Because

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Table 1
Characteristics of children at baseline and follow-up.

	Baseline			Follow-up		
	Controls	Early swimmers	*P*	Controls	Early swimmers	
N	56	140		56	140	
Boys, *N* (%)	30 (53.6)	77 (55.0)	0.86			
Age, mean ± SD, years	5.78 ± 0.43	5.64 ± 0.34	0.02	7.54 ± 0.42	7.45 ± 0.35	0.08
BMI, mean ± SD, kg/m^2	21.2 ± 3.80	20.8 ± 3.45	0.45	16.8 ± 2.24	16.7 ± 2.45	0.64
Parents						
Higher education, *N* (%)	39 (69.6)	112 (80.0)	0.12			
Parental allergies, *N* (%)	23 (41.1)	63 (45.0)	0.62			
Parental asthma, *N* (%)	7 (12.5)	18 (12.9)	0.95			
Early life care						
Smoking during pregnancy, *N* (%)	4 (7.1)	20 (14.3)	0.17			
Preterm birth, *N* (%)	12 (21.4)	35 (25.0)	0.60			
Cesarean delivery, *N* (%)	9 (16.0)	35 (25.0)	0.18			
Breastfeeding, *N* (%)	40 (71.4)	119 (85.0)	0.03			
Number of older siblings, mean ± SD	1.12 ± 1.11	0.87 ± 0.94	0.11			
Day-care attendance, *N* (%)	21 (37.5)	96 (68.6)	<0.001			
Environment and lifestyle						
Living in a rural area, *N* (%)	29 (51.8)28 (50)	54 (38.6)89 (63.6)	0.090.08	26 (46.4)28 (50)	58 (41.4)81 (74.3)	0.520.32
Living less than 100 m from a busy road, *N* (%)						
Exposure to furry pets, *N* (%)	29 (51.8)	71 (50.7)	0.89	27 (48.2)	76 (54.3)	0.44
Probiotics consumption, *N* (%)	27 (48.2)	54 (38.6)	0.22	35 (66.0)	81 (60.9)	0.51
Parental smoking at home, *N* (%)	16 (28.6)	38 (27.1)	0.84	14 (25.0)	38 (27.1)	0.76
House cleaning with bleach, *N* (%)	12 (21.4)	24 (16.1)	0.48	18 (32.1)	34 (24.3)	0.26
Chlorinated pool attendance						
Before 3 years, median (IQR), hours		33 (14.0–81.2)			33 (14.0–81.2)	
Lifetime, median (IQR), hours	26.5 (7.8–70.5)	139 (61.0–240)	<0.001	98.5 (31.5–286)	305 (166–533)	<0.001

early swimmers continued to attend more frequently chlorinated pools than their peers later during their childhood, we repeated the above analyses by testing as independent variable the time spent in chlorinated pool during the follow-up period or after the age of three years. Whether tested alone or together with the CPA before the age of three years, the attendance at chlorinated pools during the follow-up or after the age of three years was never retained as a significant predictor for any studied outcome at a *p*-value threshold of 0.20. Last, we examined whether theses risks were different when attending indoor or outdoor chlorinated pools. Given the rather limited size of our population, the numbers of subjects having attended exclusively each type of pool before the age of 3 were small. Nevertheless, it can be seen from Table 5 that odds for new-onset HDM sensitization and increased eNO were increased by attending outdoor or indoor chlorinated pools (although not always significantly because of the low numbers). As observed when combining both types of pools, associations persisted when excluding children with eczema at baseline and they were noticeably strengthened by the exclusion of children with parental allergies.

Discussion

The results of this study show that early-life attendance at chlorinated pools increases dose-dependently the risks of HDM sensitization and of airways inflammation during childhood.

Table 2
Prevalences and adjusted odds ratios for aeroallergen sensitization, airways inflammation and allergy or respiratory diseases in controls and early swimmers at the mean age of 5.7 years.

	Controls (*N* = 56)	Early swimmers (*N* = 140)	Adjusted ORs (95% CI)	*P*
Sensitization to aeroallergens				
At least one aeroallergen [a]	17 (30.4)	37 (26.4)	0.90 (0.43–1.86)	0.77
House dust mite [b]	8 (14.3)	19 (13.6)	1.12 (0.44–2.82)	0.81
Pollen [c]	9 (16.1)	25 (17.9)	1.18 (0.48–2.93)	0.72
Cat [d]	7 (12.5)	12 (8.6)	0.78 (0.28–2.21)	0.64
Airway inflammation				
Exhaled NO>15 ppb [e]	13 (23.2)	17 (12.1)	0.45 (0.19–1.09)	0.08
Allergic and respiratory diseases				
Eczema [f]	9 (16.1)	45 (32.1)	2.91 (1.23–6.89)	0.02
Allergic rhinitis [g]	8 (14.3)	16 (11.5)	0.79 (0.29–2.09)	0.63
Hay fever [h]	7 (12.5)	13 (9.3)	0.64 (0.23–1.79)	0.39
Asthma [i]	6 (10.7)	10 (7.1)	0.55 (0.17–1.77)	0.32
Bronchiolitis [j]	12 (21.4)	47 (33.6)	1.68 (0.73–3.86)	0.22

Letters indicate covariates for which ORs were adjusted.
[a] Preterm birth.
[b] Preterm birth, older siblings, probiotics, furry pets.
[c] Preterm birth, living less than 100 m from a busy road, day care attendance, gender, parental smoking.
[d] Breastfeeding, parental smoking, furry pets.
[e] Preterm birth, furry pets, older siblings, day care attendance, raw milk.
[f] Older siblings, parental smoking, furry animals, mold in bedroom.
[g] Parental asthma, parental allergies, furry pets, day care attendance, bleach, doubled-glazed windows.
[h] Preterm birth, BMI, bleach, parental asthma.
[i] Preterm birth, parental smoking, parental asthma.
[j] BMI, cesarean delivery, probiotics, living less than 100 m from a busy road, breastfeeding, day care attendance, parental asthma.

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Table 3
Prevalences and adjusted odds ratios for aeroallergen sensitization, airways inflammation and allergic diseases in controls and early swimmers at the mean age of 7.7 years.

	New-onset cases				All cases			
	Controls	Early swimmers	Adjusted OR (95% CI)	*P*	Controls	Early swimmers	Adjusted OR (95% CI)	*P*
Allergic sensitization								
At least one aeroallergen [a]	9/39 (23.0)	39/103 (37.9)	1.44 (0.70–2.95)	0.32	17/56 (30.4)	51/140 (36.4)	1.39 (0.70–2.75)	0.34
House dust mite [b]	7/48 (14.6)	35/121 (28.9)	2.93 (1.14–7.55)	0.03	11/56 (19.6)	43/140 (30.7)	2.15 (0.98–4.71)	0.056
Pollen [c]	9/47 (19.1)	10/115 (8.7)	0.40 (0.15–1.09)	0.07	11/56 (19.6)	16/140 (11.4)	0.49 (0.22–1.26)	0.11
Cat [d]	5/49 (10.2)	10/128 (7.8)	1.04 (0.28–3.81)	0.95	6/56 (10.7)	10/140 (7.1)	0.74 (0.23–2.33)	0.60
Airway inflammation								
Exhaled NO>15 ppb [e]	5/43 (11.6)	37/123 (30.1)	4.54 (1.48–13.9)	0.008	13/56 (23.2)	47/140 (33.6)	1.74 (0.81–3.72)	0.15
Allergic diseases								
Eczema [f]	3/47 (6.4)	4/95 (4.2)	0.80 (0.14–4.57)	0.80	6/56 (10.7)	24/140 (17.1)	1.60 (0.57–4.50)	0.38
Allergic rhinitis [g]	2/48 (4.2)	3/123 (2.5)	0.49 (0.07–3.21)	0.45	3/56 (5.4)	9/139 (6.5)	1.72 (0.37–7.92)	0.49
Hay fever [h]	5/49 (10.2)	7/127 (5.5)	0.75 (0.20–2.83)	0.67	10/56 (18.2)	14/140 (10.0)	0.55 (0.22–1.37)	0.20
Asthma [i]	2/50 (4.0)	2/130 (1.5)	0.35 (0.05–2.67)	0.50	5/56 (8.9)	9/140 (6.4)	0.79 (0.21–2.97)	0.73

Letters indicate the covariates for which ORs were adjusted.

[a] New-onset cases: mold in bedroom, gender, probiotics; all cases: probiotics.
[b] New-onset cases: preterm birth, mold in bedroom; all cases: probiotics, mold in bedroom, living less than 100 m from a busy road.
[c] New-onset cases: probiotics; all cases: house cleaning with bleach, probiotics.
[d] New cases: probiotics, mold in bedroom, urban area, double-glazed windows, bleach, day care attendance, smoking during pregnancy, parental asthma; all cases: bleach, probiotics; day care attendance.
[e] New cases: preterm birth, older siblings, mold in bedroom, house cleaning with bleach, smoking during pregnancy, parental allergies, parental smoking; all cases, urban area, house cleaning with bleach, cesarean delivery, preterm birth, parental allergies, parental smoking.
[f] New cases: BMI, bleach, gender, parental allergies; all cases, bleach, smoking during pregnancy, parental allergies, rural vs urban.
[g] New cases, birth at term, furry pets; all cases, mold, traffic pollution, gender, parental asthma.
[h] New cases, BMI, rural vs urban, day care; all cases: parental asthma, furry pets, parental smoking, living less than 100 m from a busy road, rural vs urban, probiotics.
[i] New cases, parental asthma; all cases, parental asthma, BMI, day care, birth at term, probiotics.

Children having spent more than 30 h in chlorinated pools before the age of three, i.e. the equivalent of a swimming session fortnightly, were respectively 3.3 and 4.7 times more likely to be sensitized to HDM and to have elevated eNO than their peers who had never attended chlorinated pools at the same age. Odds of developing these two outcomes increased up to 3.6 and 5.9, respectively, when the CPA before the age of three exceeded 60 h, which roughly corresponds to a weekly swimming session. These respiratory effects were largely independent of each other and also of traditional risks factors of allergic diseases, including parental allergies and eczema.

Our study confirms the observations of cross-sectional studies suggesting that the early attendance at chlorinated pools increases the risk of HDM sensitization (Bernard, 2007; Bernard et al., 2008; Jacobs et al., 2012). Selection bias is unlikely to explain our results as at baseline early swimmers and their controls were well matched for HDM sensitization as well as for the parental history of allergic diseases. Regarding the risk of elevated eNO, the situation is less clear-cut in that early swimmers at baseline showed a non-significant trend toward a lower prevalence for this outcome. Explanation for this difference, which persisted after adjustment for all possible confounders, is unclear. This difference might arise by chance but it might also be linked to early swimming since a decrease of exhaled NO has been observed after a swimming session in a chlorinated pool (Carbonnelle et al., 2008). This would mean that exhaled NO could present a biphasic response to chlorinated products, an early decrease due to the inhibition of NO production followed after prolonged exposure by an increase as a consequence of the airways inflammation. The case for implicating chlorinated pools in the higher prevalence of eczema in early swimmers at baseline is by contrast much stronger as this has already been reported in two cross-sectional studies (Font-Ribera et al., 2009; Chaumont et al., 2012). Furthermore, it is well known that chlorine and its by-products can irritate the skin and cause epithelial barrier defects that might facilitate skin sensitization or exacerbate eczema symptoms (Chaumont et al., 2012).

Our findings also highlight the importance of conducting prospective studies and above all of using appropriate exposure and outcome indicators when investigating the respiratory effects of chlorinated pools. The comparison between early swimmers and non-swimmers at the mean age of 5.7 or 7.5 years did not show any significant increase in the prevalences of allergic sensitization or elevated eNO in early swimmers, despite the fact that risks for these two outcomes were multiplied by 3 to 4 in the course of the follow-up. But even in the prospective analysis of our data, effects of early swimming would have also passed undetected with endpoints based on doctor-diagnosed asthma or on the prevalence of atopy defined as sensitization to any airborne allergen. With respect to exposure, our study confirms the importance of a very precise estimation of CPA during early life as associations with allergic sensitization and other outcomes emerge after only a few tens hours of CPA. To be accurate, the estimated CPA must take into account the time spent in both indoor and outdoor chlorinated pools as chlorine-compounds irritating the airways of swimmers are present in the water and in the air at the surface of both types of pools. These critical issues regarding exposure and outcome indicators might explain the negative findings of some studies. For instance, the prospective study of Font-Ribera et al. (2011) based on data collected in the 1990s did not include any specific question about the attendance of chlorinated pools before the age of 4. Before the age of 4 years, children were thus classified as ever swimmer only when in open answer to a question about “other activities” the mother wrote “swimming” (information given in the supplemental material). The estimation of CPA between age of 4 and 7 was probably also incomplete as assessment was based only on swimming during term, thus with school. In addition, this prospective study did not look specifically to the risks of sensitization to HDM, the only type of allergy that so far has been associated with early swimming. These considerations might also apply to another study of the same group (Font-Ribera et al., 2009), in which attendance at outdoor or indoor swimming pools were examined independently of each other and not combined or mutually excluded in order to avoid exposure misclassification. Of course, inconsistencies between studies might be simply related to differences in exposure levels as standards for managing water and air quality of swimming pools greatly vary between countries (WHO, 2006). Observations in Germany (Schoefer et al., 2008) may

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Table 4
New-onset cases of sensitization to HDM and of increased exhaled NO according to the total time spent in chlorinated pools before the age of three years in all children and in children with no eczema at baseline and no parental history of allergic diseases.

	All		No eczema at baseline		No parental history of allergies	
	n/*N* (%)	Adjusted OR (95% CI)	*n*/*N* (%)	Adjusted OR (95% CI)	*n*/*N* (%)	Adjusted OR (95% CI)
New-onset HDM sensitization[a]						
Never	7/48 (14.6)	1.00	4/41 (9.8)	1.00	4/28(14.3)	1.00
1–30 h	15/57 (26.3)	2.60 (0.91–7.38)	8/39 (20.5)	2.38 (0.65–8.78)	7/33 (21.2)	2.35 (0.53–10.4)
>30 h	20/64 (31.3)	3.27 (1.18–9.07)	15/47 (31.9)	4.08 (1.21–13.7)	13/37 (35.1)	4.12 (1.00–17.0)
P for trend	*0.047*		*0.01*		*0.049*	
1–60 h	21/76 (27.6)	2.63 (0.98–7.12)	12/52 (23.1)	2.63 (0.77–8.98)	11/46 (23.9)	2.55 (0.63–10.3)
>60 h	14/45 (31.1)	3.60 (1.21–10.7)	11/34 (32.4)	4.46 (1.24–16.0)	9/24 (37.5)	5.02 (1.11–22.7)
P for trend	*0.06*		*0.02*		*0.053*	
New-onset exhaled NO>15 ppb[b]						
Never	5/43 (11.6)	1.00	4/37 (10.8)	1.00	2/25 (8.0)	1.00
1-30 h	17/59 (28.8)	4.41 (1.33–14.7)	11/40 (27.5)	3.34 (0.92–12.1)	11/33 (33.3)	11.8 (1.62–85.7)
>30 h	20/64 (31.3)	4.66 (1.42–15.2)	14/45 (31.1)	3.68 (1.02–13.2)	14/37 (37.8)	15.1 (2.04–111)
P for trend	*0.03*		*0.04*		*0.01*	
1–60 h	21/78 (26.9)	3.88 (1.21–12.4)	13/51 (24.5)	2.92 (0.83–10.3)	13/45 (28.9)	9.54 (1.32–68.8)
>60 h	16/45 (35.2)	5.92 (1.72–20.5)	12/34 (35.3)	4.64 (1.23–17.5)	12/25 (48.0)	27.9 (3.36–332)
P for trend	*0.01*		*0.02*		*0.002*	

[a] Adjusted for preterm birth, mold in bedroom.
[b] Adjusted for preterm birth, older siblings, mold in bedroom, bleach, smoking during pregnancy, parental allergies, parental smoking.

also be explained by the fact that standards for free and combined chlorine (0.3–0.6 and <0.2 mg/l, respectively) are much lower in Germany than countries where respiratory effects of swimming pools have been reported.

The explanation for the different patterns of effects associated with chlorinated pools probably lies in the fact that allergic diseases develop in several steps, each step requiring a certain time to be realized, depending on the exposure to the triggers and the natural course of the disease. Current evidence suggests that chlorine-based irritants in swimming pools promote the development of allergic diseases by two different modes of actions (MOA). The first MOA is an interaction with atopic status promoting the clinical expression of allergic rhinitis and asthma. This interaction, which by definition can be triggered only in swimmers sensitized to aeroallergens, requires a CPA of at least several hundreds of hours. Such a CPA is unlikely to be achieved before adolescence in most recreational swimmers, explaining why early swimming was not associated with clinically diagnosed allergic diseases in very young children, in our study as well as in studies conducted by others (Schoefer et al., 2008 Font-Ribera et al., 2009, 2011). The second mode of action is an interaction between chlorinated pool attendance and allergen exposure, which promotes allergic sensitization. This second MOA can be triggered in early life after a CPA of a few tens of hours. However, to be effective, this MOA requires 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. This probably explains why the increased HDM sensitization associated with early swimming is observed after a time lag time of a few years and also why sensitization to pollen is not increased in early swimmers. Differences in the exposure patterns between these allergens probably also explain why remission of sensitization observed during the follow-up was more frequent with pollen than with HDM. Even though they operate at different stages of the allergic march, these two MOA probably share the same basic mechanism i.e. which is a disruption of the airways epithelial barriers by chlorine-based oxidants (Bernard et al., 2003; Bernard, 2007; Carbonnelle et al., 2002; Lagerkvist et al., 2004; Bougault et al., 2012). The types of epithelial defects in epithelial barrier enhancing allergic sensitization and its clinical manifestations are largely unknown. There is, however, some evidence from biomarker studies that epithelial defects promoting allergic sensitization might consist in an increased epithelial hyperpermeability facilitating the allergen delivery to dendritic cells associated with a dysfunction or dedifferentiation of epithelial cells secreting immunomodulatory proteins, in particular the Clara cells (Sardella et al., 2012a,b).

Our study has some limitations. The main limitation is the relatively small size of the cohort and in particular of the group used as reference. Because of the increasing popularity of swimming in affluent countries and the widespread use of chlorine to sanitize swimming pools, it has become increasingly difficult to recruit children having never attended chlorinated pools during early life. These limitations, however, have been partly compensated by the prospective design of our study and the use of accurate and sensitive effect and exposure indicators. Another limitation is the lack of data regarding the concentrations of chlorine and its by-products in the outdoor and outdoor swimming pools attended by the children. By contrast, the fact that tap water in Belgium is disinfected with very low level of chlorine has probably eliminated a source of confounding. These methodological strengths along with the relatively high prevalences of studied outcomes explain why our study could evidence significant exposure-response relationships despite a relatively limited sample size.

In conclusion, attendance at chlorinated swimming pools in early life is associated independently with higher risks of developing IgE sensitization to HDM and an increased exhaled NO, which

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Table 5
New-onset cases of sensitization to HDM and of increased exhaled NO associated with the attendance at indoor or outdoor chlorinated pools before the age of three years, in all children and in children with no eczema at baseline or with no parental history of allergic diseases.

	Attendance at chlorinated swimming pools before the age of three years							
	Never		Indoor pools only			Outdoor pools only		
	n/*N* (%)	OR	*n*/*N* (%)	OR (95% CI)	*P*	*n*/*N* (%)	OR (95% CI)	*P*
New-onset HDM sensitization[a]								
All subjects	7/48 (14.6)	1.00	11/40 (27.5)	2.70 (0.88–8.25)	0.08	9/30 (30.0)	3.06 (0.94–10.0)	0.06
No eczema at baseline	4/41 (9.8)	1.00	7/20 (35.0)	2.66 (0.69–10.2)	0.16	7/24 (29.2)	5.15 (1.24–21.3)	0.02
No parental allergies	4/28 (14.3)	1.00	7/24 (29.2)	3.10 (0.68–13.5)	0.15	4/17 (23.5)	2.71 (0.49–14.8)	0.25
New-onset exhaled NO>15 ppb[b]								
All subjects	5/43 (11.6)	1.00	17/40 (42.5)	8.36 (2.27–30.8)	0.001	5/28 (17.8)	1.90 (0.43–1.50)	0.40
No eczema at baseline	4/37 (10.8)	1.00	12/29 (41.4)	8.04 (1.87–34.6)	0.005	4/20 (20.0)	1.64 (0.32–8.28)	0.55
No parental allergies	2/25 (8.0)	1.00	11/13 (84.6)	60.0 (3.0–1400)	0.008	5/14 (35.7)	27.2 (1.45–509)	0.03

[a] Adjusted for preterm birth, mold in bedroom.
[b] Adjusted for preterm birth, older siblings, mold in bedroom, bleach, smoking during pregnancy, parental allergies, parental smoking.

are among the strongest predictors of rhinitis and asthma (Olin et al., 2010; Malinovschi et al., 2012). If one adds to these findings observations from previous studies showing that pool chlorine interacts with atopic status to increase the risks of eczema, allergic rhinitis and asthma, this suggests that exposure to chlorine-based oxidants in swimming pools is a potent driver of the allergic march, promoting IgE sensitization at an early stage and later the clinical manifestations of atopy. These findings stress the importance of minimizing exposure of young children to chlorine and its by-products in swimming pools by a strict regulation of these chemicals or the use of alternative disinfection methods.

Author contributions

CV recruited the children, organized their examination in schools, performed the collection and analysis of data and contributed to the writing of the manuscript. AS was responsible for the Rhinostick tests both in the field and in the laboratory. AB provided the scientific guidance of the project, participating to the study design, the data analysis and interpretation and the writing of the manuscript.

Conflict of interest

None of the authors has a conflict of interest to disclose.

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Reply

From the Authors:

We are grateful for the interest Dr. Gupta and colleagues have shown in our manuscript and its implications (1). We of course agree that minimal clinically important difference estimates are context dependent. In this study, we reported differences based on 6-minute walk (6MW) 1 year apart without treatment change, because ECLIPSE was a prospective observational study (2). This context is of course explicit in the METHODS. We believe our work is a useful addition to the literature because prior studies have been context dependent in a different sense, specifically around improvement rather than natural progression and around a treatment intervention, typically rehabilitation and/or surgery, to which the patient cannot be blinded.

The question as to whether different thresholds for change in 6-minute-walk distance (Δ6MWD) produce different odds ratios for mortality is interesting. In the original article, we had already given data for −30 and −39.6 m. As one would expect, lowering this threshold adds patients who don't die to the group, with the larger Δ6MWD thus diminishing the hazard ratios (HRs) and weakening statistical significances, though these are retained by virtue of the very large sample size (−20 m: HR = 1.87, 95% confidence interval, 1.24–2.81, $P = 0.003$; −25 m: HR = 1.90, 95% confidence interval, 1.27–2.85; $P = 0.002$). Nevertheless this question fails to appreciate the origin of the −30 m, which is the difference in Δ6MWD, rounded from −29.7 m, between those who died and those who did not.

Lastly, we accept that in a literal sense, death cannot be considered a minimal change. However death is clearly a categorical variable; like pregnancy, one cannot be "slightly" dead. Against that background and in the context of the work, we believe the use of the word is justified. We do agree that minimal clinically important difference for perceptible improvement in symptoms, as Gupta and colleagues propose, has value in relation to some therapeutic interventions, and particularly for conditions where death is infrequent. Nevertheless, because subtypes of COPD do have a poor prognosis (3–5) and COPD is not just a disease affecting the symptoms domain, we also submit that a clinical assessment tool, like 6MW, that detects mortality risk will be of value both to clinicians and those designing clinical trials.

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Allergic Sensitization and Airway Inflammation after Early Swimming

To the Editor:

There is increasing evidence that irritant chlorination products in swimming pools can cause respiratory problems in pool workers and competitive swimmers (1, 2). Whether these risks may extend to recreational swimmers is an important question which to date remains unresolved. Several studies of adults and adolescents (3–8) have shown that recreational swimming in outdoor or indoor chlorinated pools is associated with increased risks of allergic diseases, in particular asthma. In adolescents, the asthma risk associated with chlorinated pool attendance appears to largely be the consequence of an interaction with atopy, increasing only among subjects sensitized to aeroallergens (6, 7). Other studies found no increase of asthma risk with swimming pool attendance (8, 10). These studies, however, were more focused on children who perhaps were too young for detecting among them associations with atopic asthma, a chronic disease developing mostly during late childhood (11). Further complicating the matter, some observations suggest that early exposure to chlorinated pools might promote allergic sensitization and thus contribute to the development of atopy itself. Attendance at chlorinated pools during infancy or early childhood has indeed been associated with an increased risk of eczema (9) and house dust mite (HDM) sensitization (12, 13), two outcomes that are predictive of rhinitis and asthma later in life.

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Author Contributions: C.V. recruited the children, organized their examination in schools, performed the collection and analysis of data, and contributed to the writing of the manuscript. A.S. was responsible for the screening of aeroallergen-specific IgE in nasal mucosa. A.B. provided the scientific guidance of the project, participating in the study design, the data analysis and interpretation, and the writing of the manuscript.

TABLE 1. NEW-ONSET CASES OF SENSITIZATION TO HDM AND OF INCREASED EXHALED NO ACCORDING TO THE TOTAL TIME SPENT IN INDOOR OR OUTDOOR CHLORINATED SWIMMING POOLS BEFORE THE AGE OF 3 YEARS

	Cumulative attendance at chlorinated pools before the age of 3 yr (h)				
	Never	1–30	>30	1–60	>60
New-onset HDM sensitization*					
n/N (%)	7/48 (14.6)	15/57 (26.3)	20/64 (31.3)	21/76 (27.6)	14/45 (31.1)
Crude OR (95% CI)	1.00	2.09 (0.77–5.66)	2.66 (1.02–6.96)	2.24 (0.87–5.76)	2.65 (0.95–5.76)
Adjusted OR (95% CI)	1.00	2.60 (0.91–7.38)	3.27 (1.18–9.07)	2.63 (0.98–7.12)	3.60 (1.21–10.7)
P for trend			0.047		0.06
New-onset exhaled NO > 15 ppb†					
n/N (%)	5/43 (11.6)	17/59 (28.8)	20/64 (31.3)	21/78 (26.9)	16/45 (35.6)
Crude OR (95% CI)	1.00	3.08 (1.04–9.15)	3.46 (1.18–10.1)	2.80 (0.97–8.07)	4.19 (1.38–12.8)
Adjusted OR (95% CI)	1.00	4.41 (1.33–14.7)	4.66 (1.42–15.2)	3.88 (1.21–12.4)	5.92 (1.72–20.5)
P for trend			0.03		0.01

Definition of abbreviation: CI = confidence interval; HDM = house dust mite.

N represents the number of subjects in the different groups that at baseline were not sensitized to HDM or were negative in the exhaled nitric oxide test.

*Adjusted for preterm birth, mold in bedroom.

† Adjusted for preterm birth, number of older siblings, mold in bedroom, use of bleach for house cleaning, smoking during pregnancy, parental allergies and parental smoking.

We here report the results of a 2-year prospective study comparing the development of new-onset allergic sensitization and diseases and increased exhaled nitric oxide (eNO) between children who started to swim in early life and those who did not.

The study was conducted among 196 schoolchildren in 30 schools located in the areas of Brussels and Liège in Belgium. The first examination took place between December 2007 and March 2008 when children were all in the third year of kindergarten (mean age, 5.7 yr; SD, 0.37). The children were then reexamined 2 years later by visiting the schools in the same order between December 2009 and March 2010. The baseline group included 431 children, but 2 years later, 128 of them had left the schools. Among the remaining children, 236 (77.9%) participated in the study with the agreement of their parents and 196 of them successfully performed all of the tests. Subjects and nonsubjects did not differ significantly regarding studied outcomes, chlorinated pool attendance, and the main risk factors of allergies. Parents filled out a self-administered questionnaire about the health of their child and factors likely to influence the development of respiratory and allergic diseases. The questionnaire included questions intended to calculate the total time (cumulative pool attendance [CPA]) the child had spent in indoor or outdoor chlorinated pools before the age of 3 (referred to hereinafter as "early swimmers"), during the 2-year follow-up, and over their lifetime. The examination of children, performed in schools between 9:00 A.M. and 1:00 P.M., included a screening of aeroallergen-specific IgE in nasal mucosa by the Rhinostick test (14) and the measurement of eNO (NIOX analyzer; Aerocrine AB, Solna, Sweden). The Rhinostick test was calibrated with serum standards and was considered positive at specific IgE $\geqslant$ 0.35 kIU/L (14). For eNO, we used a cutoff of 15 ppb (15), which in our study corresponded to the 95th percentile of baseline values in children who were nonatopic. The study protocol was approved by the Institutional Review Board of the Catholic University of Louvain. We used backward logistic regression analyses to calculate the crude and adjusted odds ratios (aORs) of outcomes according to increasing CPA while adjusting for confounders that remained in the model at a P value < 0.20. For these analyses, we used children who never attended chlorinated pools before the age of 3 as referents and we dichotomized early swimmers at CPA cutoff values (30 or 60 h) that corresponded approximately to the median (32 h) or twice the median of their CPA. Because there were only 10 children who never visited chlorinated pools, the CPA during the follow-up or over the child's lifetime was stratified in tertiles. Results were considered statistically significant at a P value < 0.05.

Early swimmers and their controls were well matched with respect to gender, parental history of allergies, and other traditional risk factors of atopic diseases. At baseline, the two groups did not show any significant difference in the prevalence of increased eNO and of allergic sensitization or diseases, except for eczema, which was much more frequent in early swimmers (aOR, 3.17; 95% confidence interval [CI], 1.29–7.81; $P = 0.01$). When children were reexamined 2 years later, we found no significant differences in the prevalence of new-onset allergic diseases between the two groups. By contrast, there was a marked increase in the prevalence of new-onset sensitization to HDM (aOR, 2.93; 95% CI, 1.14–7.55; $P = 0.03$) and new-onset increased eNO (aOR, 4.54; 95% CI, 1.48–13.9; $P = 0.008$) among early swimmers as compared with controls. As shown in Table 1, the adjusted ORs for these two outcomes increased dose dependently with the time spent in indoor or outdoor chlorinated pools before the age of 3. These associations were independent of each other, of parental allergies, and of preexisting diseases, including eczema. Such associations were not seen with IgE sensitization to pollen or cat allergens. Whether tested alone or with the CPA before the age of 3, the CPA during the follow-up (median [h] in early swimmers, 161; in controls, 77) or over the child's lifetime (early swimmers, 309; controls, 99) was never retained as a significant predictor for any of the studied outcomes.

This prospective study shows that children who regularly attended indoor or outdoor chlorinated pools during their infancy were more likely to develop airway inflammation and a sensitization to HDM. Attendance at chlorinated pools later during childhood did not emerge as a significant predictor for these risks. These specific associations with early swimming, also observed in cross-sectional studies (12, 13), suggest, not surprisingly, that developing airways of young children are highly vulnerable to injury by chlorination products. Our study has some limitations, in particular regarding the exposure assessment on the basis of the questionnaire and the lack of a control group attending nonchlorinated pools. However, because at the age of 3, and even later, children cannot really swim and do not actively practice a sport, physical exercise is unlikely to be the cause of our findings. The most plausible explanation is that the exposure to chlorine-based irritants in swimming pools causes defects in epithelial barriers predisposing children to allergic sensitization and airway inflammation (11, 12). To enhance sensitization, these epithelial defects logically require a concomitant and sufficient exposure to allergens, a condition more easily met with perennial allergens like HDM than with seasonal allergens linked to pollination. This probably explains why sensitization

to pollen was not increased in early swimmers and also why the risk of HDM sensitization increased only after a time lag of a few years. Combined with data from previous studies showing that pool chlorine can interact with atopic status to increase the risks of allergic rhinitis or asthma (6, 7), our findings suggest that chlorine used to disinfect swimming pools is a significant driver of the atopic march, promoting allergic sensitization at an early stage and later the clinical manifestations of atopy.

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Anomalous Origin of the Left Coronary Artery from the Pulmonary Artery Presenting as Sudden Death in a Young Woman



To the Editor:

Sports-related sudden death is a specific nosological entity, occurring in the vast majority of cases without prodrome. Its incidence in France is currently 4.6 per million per year, affecting males in 95% of cases (1). In addition, under the age of 35, ventricular arrhythmia is the leading cause of sudden death, followed by coronary artery diseases and cardiomyopathy (2).

We present the case of a 16-year-old woman admitted for a sports-related sudden cardiac arrest leading to diagnosis of abnormal implantation of the coronary arteries.

A 16-year-old woman, measuring 1.65 m and weighing 56 kg, with no previous abnormal medical history collapsed during school running practice without prodromal symptoms. A diagnosis of cardiac arrest was made and cardiopulmonary resuscitation (CPR) was performed by the sports teacher (2 min of mouth-to-mouth breathing followed by external cardiac massage). The emergency services arrived 5 minutes after collapse. Their semiautomatic defibrillator advised an external electric shock (pace recovered *a posteriori* showed ventricular fibrillation), which was successful with return to sinus rhythm and spontaneous circulation. Due to persistent coma (Glasgow Coma Score = 5) and after initial CPR, the patient was intubated by the Mobile Emergency and Resuscitation Service's physician, who arrived 20 minutes after the initial loss of consciousness. The patient was then transferred to the intensive care unit for further care and etiological appraisal. Computed tomography (CT) of the brain showed no abnormality, echocardiography didn't find any systolic–diastolic dysfunction, and ECG revealed anterior and lateral ST segment elevation associated with a high maximum peak of troponin Tc of 150 ng/L (n < 14 ng/L) to H6. No metabolic abnormality was found except for lactic acidosis (lactates, 3.44 mmol/L; pH 7.24), which was quickly reversible. Evolution was favorable in the intensive care unit with an adapted revival without neurological deficit (Cerebral Performance Category score = 1) after 24 hours of therapeutic hypothermia at 33°C with a CoolGuard device (IST Cardiology, Saint-Contest, France). The patient was extubated 3 days after admission (D3) and then transferred to the cardiology department for etiological appraisal at D4.

Cardiac CT angiography showed agenesis of the common trunk of the left coronary artery with fistula between the proximal left anterior descending artery and pulmonary artery. This was associated with dilatation of the coronary arteries, constituting a significant distal and intraseptal left–right anastomotic network (Figure 1). Cardiac magnetic resonance imaging (MRI) showed a left-to-right shunt (via an anastomosis between the right coronary artery and the left coronary artery) of 1.4 L/minute and a subendocardial infarction in the lateral wall of the left ventricle. Left ventricular ejection fraction was maintained at 63%. The diagnosis was anomalous origin of the left coronary artery from the pulmonary artery (ALCAPA) (also known as Bland-White-Garland syndrome). The patient underwent cardiac surgery with reimplantation of the left coronary artery into the aorta. The postoperative course was uneventful, allowing hospital discharge at D18 with β-blocker and angiotensin-converting enzyme inhibitor. Six months after initial admission, the patient suffered no neurological or cardiac sequelae, enabling resumption of sports with no restrictions.

ALCAPA, which corresponds to implantation of the left coronary artery into the pulmonary artery, is a rare congenital heart