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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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-September 2013-

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Acknowledgments

Came at the end of this path I can’t help but thank all those who have accompanied and supported me during these years.

Professor Alfred Bernard for giving me the opportunity to make this experience and for the professional and scientific growth gained over the years in his laboratory. I would also like to thank him for having understood the difficulty of making a research project, working part-time, and for not making the "absences", due to my teaching activities, weigh upon me.

Professor Marie-Christine Many because without her this adventure would never have begun, because she wanted to believe in a young Italian woman 8 years ago. I thank her for the great professional and human support that she has always given me and for the passion for teaching that she was able to convey to me.

The president and the members of the jury for their valuable advices and suggestions that have allowed me to improve the quality of my work.

The team of Toxicology, with whom I had the pleasure of working in the laboratory and "on the road": Xavier, Catherine, Virginie, Agnès, Fred, Laurent, thank you!
I would like to make a special thanks to Catherine, Laurent, Xavier and Virginie, because their presence has been dear and precious to me during all this time.

Professor Jean-Baptiste Demoulin, Professor Jean-François Denef and the whole team of teachers, assistants, technicians and secretaries of histology

and biology: thanks to you I will always keep a wonderful memory of the hours spent explaining how to calculate a scale model and discovering that a *Lumbricus* can reach a length of three meters, that the uterus is part of the intestine, that the taste buds are located in the fingertips, etc..

Marc, Vincent and Jonathan for helping me with the histological analysis.

My students, "mixed blessing" of these 8 years.

My father and my mother for their love and being by my side despite the kilometers that separate us.
My sister, for being always my accomplice…

The people who were and are close to me every day in Brussels: Brad, Tina, Gloria, Angela Agnelli, Dario, Barbara and many others.

Cathal for the good times in rue Général Fivé and for correcting my English.
Philippe for bringing me on the crest of the wave!

All my friends for being my family.

Table of contents

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).

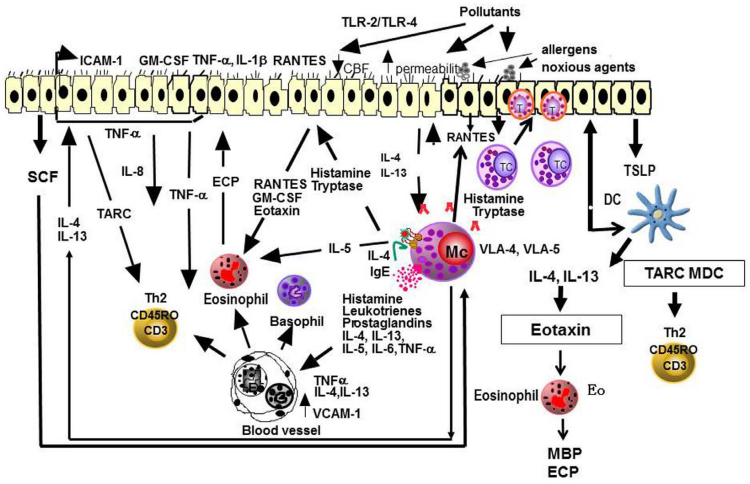

Figure 1: Ongoing inflammation in allergic rhinitis (from Pawankar R et al, 2002).

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'.

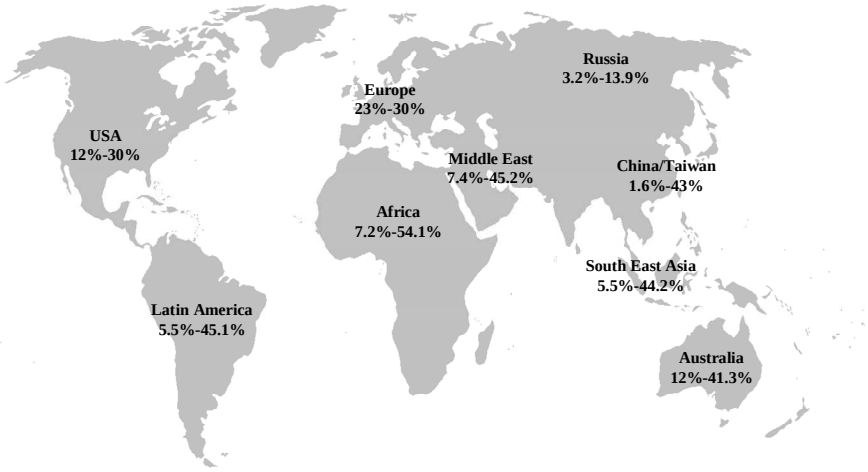

Figure 2: Prevalence of AR or AR/C in different regions of the world (adapted from Katelaris CH et al, 2012).

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

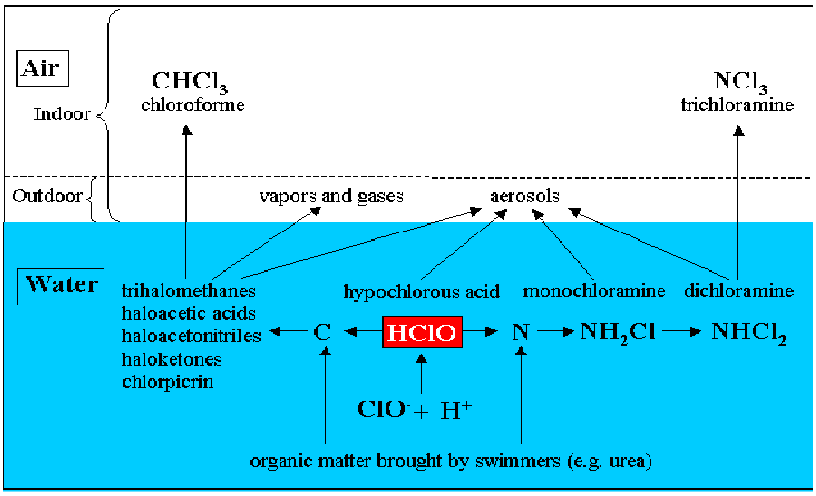

Figure 3: Main chlorination products in the water and air of indoor and outdoor pools (adapted from Voisin C and Bernard A, 2008).

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).

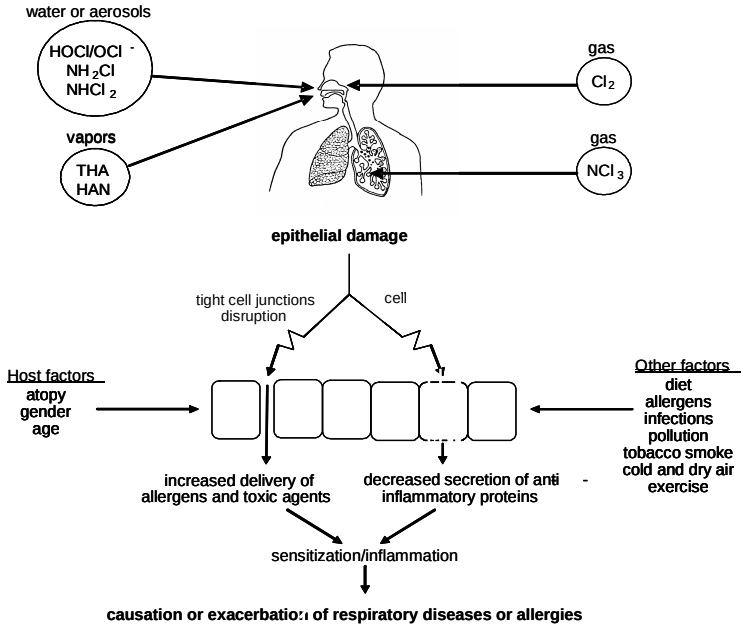

Figure 4 : Effects of chlorination by products on the respiratory epithelium.

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).

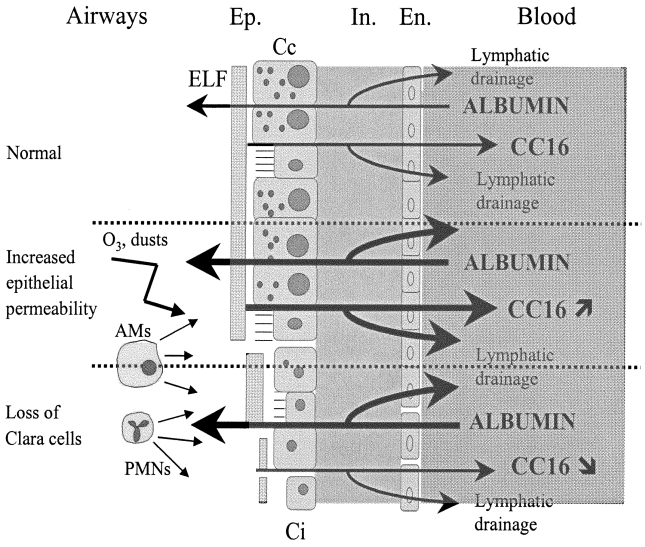

Figure 5: Schematic representation of the passage of albumin and CC16 through the epithelial barrier. En., endothelium;Ep., epithelium;In., interstitium;Cc, Clara cell;Ci, ciliated cells;ELF, epithelial lining fluid.

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.

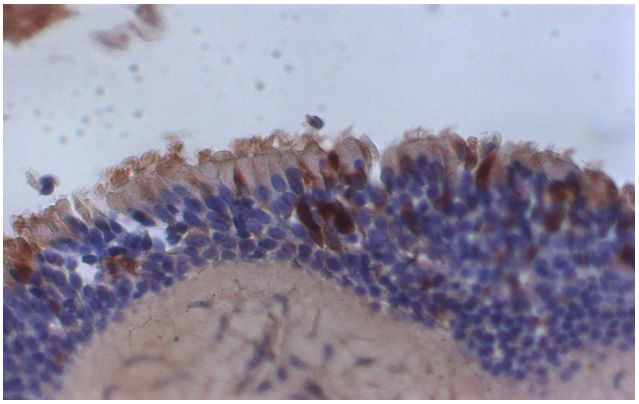

Figure 6: CC16 immunostaining in human nasal biopsy (Sardella A et al, unpublished).

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.

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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