UNIVERSITY OF PAVIA

PhD Program in Biomedical Sciences
Neuroscience Curriculum

ADENOIDAL IMMUNITY IN CHILDREN: IS THERE A CORRELATION WITH PASSIVE SMOKE, ATOPY AND "LOW DOSE MEDICINE COMPOUND"?

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To my parents

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1 INTRODUCTION

1.1 Anatomy

Anatomically, the adenoids are part of Waldeyer's ring, a lymphoid structure consisting superiorly of the pharyngeal tonsils (adenoids), laterally of the two palatine tonsils (located at the entrance of the oropharynx) and inferiorly of the lingual tonsils, sited at the base of the tongue (Casselbrant, 1999).

The upper respiratory tract-associated lymphoid tissues, comprising the adenoids and palatine tonsils, are the body's first line of immune defense and are important effector organs in both mucosal-type and systemictype adaptive immunity (Figure 1).

In particular, tonsils and adenoids play an important immune-inductive role as components of mucosa-associated lymphoid tissue (MALT) and show similarities with lymph nodes participating as effector organs of local systemic-type as well as mucosal-type of adaptive immunity. Structure and localization of adenoids influence their mechanism of immune responses and their response to external antigens, leading them to play a crucial role in the host defense against pathogens in the upper respiratory tract (Brandtzaeg P, 2003).

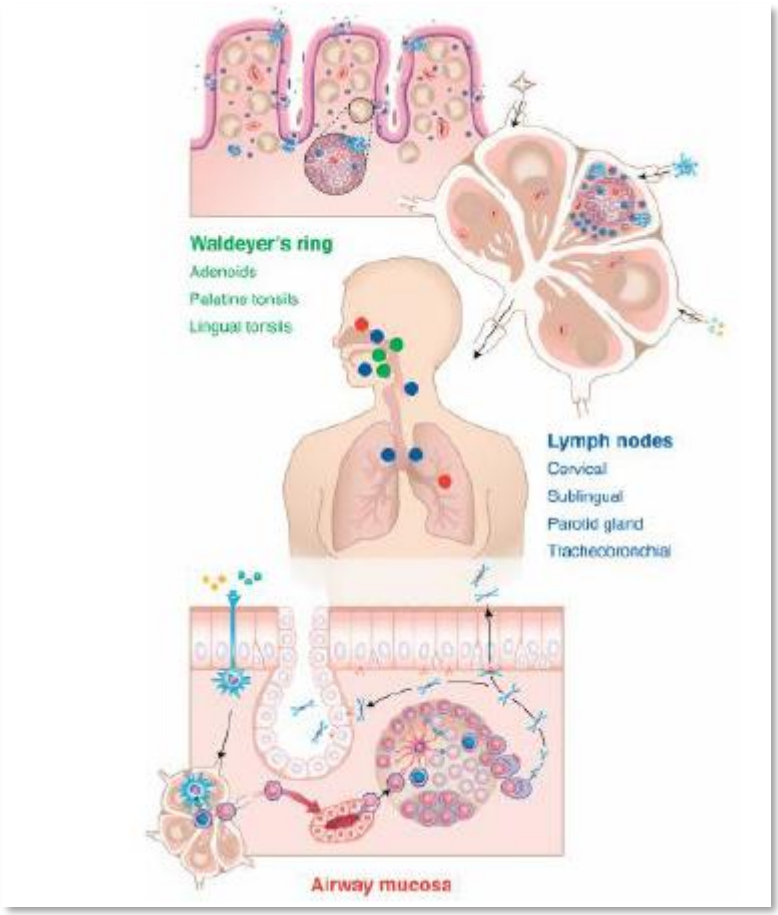

Figure 1: Upper respiratory tract-associated lymphoid tissue (Kato A et al. 2013)

Recurrent or chronic respiratory infections can induce histomorphological and functional changes in the immunological barrier of the adenoids, sometimes making surgical treatment necessary. Several different conditions can lead to adenoid hypertrophy and it is commonly assumed that exposure to passive smoking and allergic diseases contribute to recurrent episodes of respiratory inflammation in children through the reduction of IFN-γ-producing CD81 T lymphocytes; the adenoids are in fact a potential site of allergic inflammation thanks to local synthesis of total and specific IgEs by adenoid mast cells (Marseglia et al., 2011).

1.2 Histology and Immunology

Adenoids are strategically located for mediating local and regional immune functions as they are exposed to antigens from both the outside air, allergens and pathogens, and the alimentary tract (Brandtzaeg P et al, 2003). At the rear of the nasal passages, where they are primarily exposed

to inhaled and environmental antigens, the adenoids consist of flat longitudinal folds, with sero-mucous glandular ducts opening on the base and a fine and incomplete capsular structure; a ciliated columnar pseudo-stratified epithelium with goblet cells and a non-keratinous stratified epithelium cover these organs that originate from lymph node clusters in the nasal-pharyngeal wall.

The anatomical structure of adenoids (Figure 2) composed by the epithelium, follicular germinal centers (GCs) with the mantle zone and interfollicular area, participates to the modulation of their immune response. In particular, antigen uptake by M-cells in the epithelial zone plays a central role in antigens processing, leading to the generation and dissemination of antigen-specific memory effector B-lymphocytes (Türkoğlu Babakurban S et al, 2016)

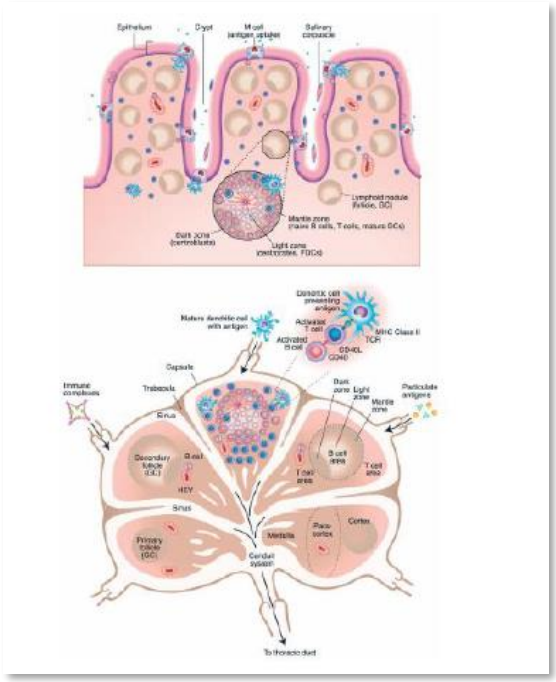

Figure 2: detailed overview of primary inductive lymphoid tissues contained in adenoids (top) and lymph nodes (bottom) (Kato A et al. 2013).

At 16 weeks of gestation primary follicles appear in human tonsils, similarly to the Peyer's patches of gut-associated lymphoid tissue (GALT). Nevertheless the early formation of GC follicles, B-cell activation take place only shortly after birth, induced by exogenous antigens, while the terminal differentiation of effector B cells to extra-follicular plasma cells begins approximately 2 weeks after birth (Fukuyama S et al, 2000).

The GCs characteristically arise in T cell-dependent B-cell responses and are associated with clonal expansion of B cells, positive selection of B cells that are able to receive antigen-specific

signals by high affinity, somatic hypermutation in B-cell immunoglobulin (Ig) variable (Ig V)-region genes and induction of J chain gene expression, essential for the formation and selective external transport of secretory IgA (sIgA) and secretory IgM (sIgM) antibodies (Brandtzaeg P et al, 1999).

Together with tonsils, adenoids seem to play a role as inductive sites not only for local immune responses to external antigens, but also for nasal vaccines and effector sites for antibody responses (Türkoğlu Babakurban S et al, 2016).

Adenoids consist of dedicated lymphoepithelial tissue and are composed of epithelial cells, lymphocytes, macrophages and dendritic cells; histologically, they are characterized by follicular germinal centers and interfollicular areas, which are predominantly populated by T-lymphocytes.

The location and function of effector T-cells are crucial for generating an effective immune response. In particular, CD81 T lymphocytes can mobilize two main mechanisms: cytolysis, and production of cytokines, chemokines and microbicidal molecules. When the production of IFN-g by Th1 adenoidal lymphocytes is reduced (for example in children exposed to passive smoking), patients become more susceptible to infectious viral diseases, which often precede upper respiratory tract infections, potentiating the replication of pathogenic bacteria in adenoidal tissue (Avanzini et al., 2008). Secretory IgA is the main antibody class in adenoidal tissue and is important in mucosal immunity, binding to bacteria and suppressing bacterial colonization of the epithelium (Figure 3). Some authors have recently pointed out that IgA expression is significantly lower than normal in the adenoids of children suffering from otitis media (OM) with effusion (Wang et al., 2012).

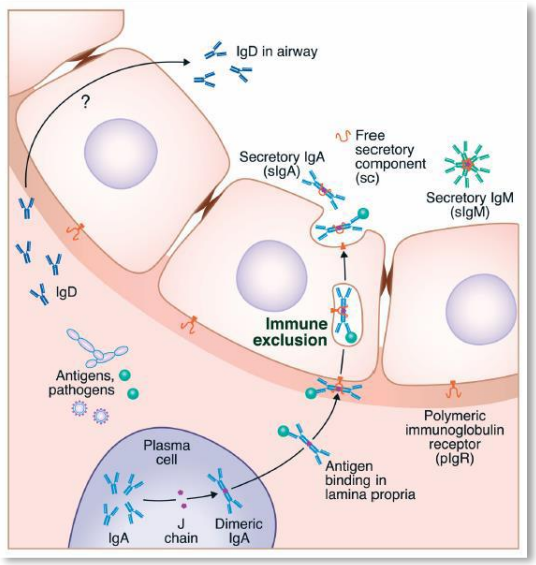

Figure 3: Local expression of immunoglobulins of the IgA an IgM isotype leveraged for the removal of antigens, allergens and pathogens via the polymeric immunoglobulin receptor (Kato A et al. 2013)

Moreover, under normal conditions, the active interaction between innate and adaptive immunity, and with nonspecific mechanical factors (such ciliary movement), potentially contribute to preventing microorganism invasion. This is partly mediated by Toll-like receptors (TLRs), which are crucial for immunological function in upper airway diseases (they are reduced, for example, in children with recurrent respiratory infections or exposed to passive smoking; Ricci et al., 2005). The important role of these proteins in the immunological and antimicrobial response is further underlined by the increased expression of TLR7 in children with OM with effusion (Granath et al., 2010). A recent study investigated the possibility that some serum mediators could serve as surrogate markers of adenoidal hypertrophy in children. In particular, high serum levels of myeloperoxidase, a typical marker of neutrophil activation, are observed in children with recurrent lower respiratory infection; increased serum eosinophilic cationic protein, a typical marker of eosinophil activation, is identified in children with recurrent upper respiratory infections; and elevated levels of CD163 glycoprotein, a typical marker of monocyte/macrophage activation, could indicate the extent of adenoidal hypertophy. In any case, the activities of these inflammatory cells are significantly higher in adenoidal hypertrophy children than healthy controls (De Amici et al., 2012).

1.3 Adenoid disorders

There are several disorders that can impact the adenoids in children (Brambilla I et al, 2014).

1.3.1 Adenoid hypertrophy

Adenoids are the first defense line against respiratory pathogens that infect upper airways organs, including nose, mouth and pharynx.

The integrity of adenoid tissue is different from one child to another and is usually maximal between 2 and 6 years of age, after which the size undergoes regression.

Adenoid tissue integrity varies among children but is usually maximal between 2 and 6 years of age, after which the size undergoes regression. As a part of the nasopharyngeal lymphoid tissue, adenoids normally provide resistance against upper respiratory tract infections (URTI) but they may per se become a source of recurring and chronic infection (Pereira et al, 2018).

Adenoid hypertrophy is clinically expressed by nightly snoring, nasal obstruction, mouth breathing, recurrent sinusitis, auditory tube dysfunction, OM, reduced ability to smell and taste, speech problems, changes in facial growth and behavioral development, and/or more serious problems such as obstructive sleep apnea syndrome. Factors contributing to sleep apnea include obesity, allergy, asthma, gastroenterological reflux disorder, abnormalities in the physical structure of the face or jaw, and various medical and neurological conditions. These complications frequently lead to a need for adenoidectomy or adenotonsillectomy, which is still one of the most common pediatric surgical procedures worldwide, to improve overall quality of life (Tűrkoglu Babakurban et al, 2016).

1.3.1.1 *Adenoiditis*

Infection is certainly the main cause of adenoid hypertrophy.

In the palatine and pharyngeal tonsils, virus and bacteria are placed within deep crypts, which become some sort of reservoirs and contribute to adenotonsillar tissue hypertrophy and Eustachian tube (ET) dysfunction (Jose Luiz Proenca-Modena et al., 2012) (Figure 4). For example, a recent study demonstrates that a very high proportion of patients with chronic adenotonsillar diseases harbors at least one respiratory virus in their adenotonsillar tissues. The most frequently detected viruses are human adenovirus in 47.1%, human enterovirus in 40.5%, human rhinovirus in 38%, human bocavirus in 29.8%, human metapneumovirus in 17.4%, and human respiratory syncytial virus in 15.7%. These specific viruses preferentially infect some

tissues more frequently than any other and their persistence/latency can induce chronic adenotonsillar diseases (Avanzini et al., 2008).

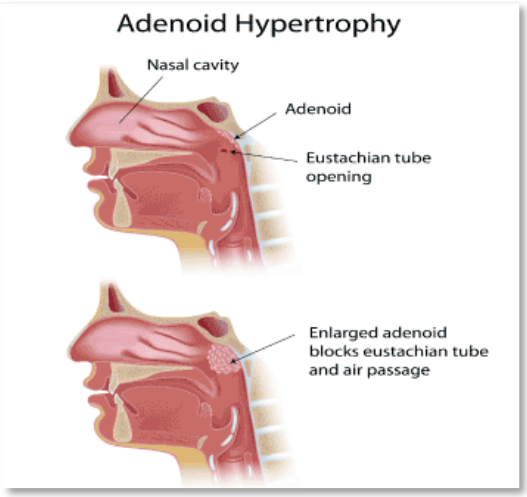

Figure 4: Obstruction caused by hypertrophic adenoid tissue

1.3.1.2 *Other conditions*

Several different conditions, such as recurrent or chronic respiratory infections, can induce histomorphological and functional changes in adenoidal structure that drive to adenoid hypertrophy often making surgical treatment necessary. In particular, in children, also allergic diseases could contribute to recurrent episodes of respiratory inflammation reducing IFN-γ producing CD8 T lymphocytes. Moreover, the adenoids are a potential site of allergic inflammation thanks to local synthesis of total and specific IgEs by adenoid mast cells (Marseglia et al., 2011).

1.3.2 Otitis media (OM)

Many studies underline the connection between the pathogenesis of OM and functional and mechanical obstruction of the ET, identified in several pathologic conditions such as bacterial/viral upper airway infections, adenoiditis, nasal inflammation including allergic rhinitis, enlarged adenoids or nasopharyngeal tumors, altered wall compliance or inefficient active/muscular opening due to a lower amount and stiffness of cartilage support and reduced muscle efficiency. Increasing attention has also been focused on the potential role of adenoids in inducing ET dysfunction in allergic patients, and different studies have revealed that the adenoid tissue contains significantly more mast cells in patients with ET dysfunction and OM with effusion (Bylander Grothet al, 1998). Biofilm-producing bacteria, for example, are detectable on adenoid

surfaces especially in children with recurrent acute OM and persistent OM with effusion. The more frequent location of bacterial biofilms near the ostium of the ET indicates not only that the adenoids are a reservoir for bacteria but also that adenoiditis can predispose to otitis and other recurrent upper airways infections such as rhinosinusitis.

Moreover, anatomical obstruction of the ET and worsen middle ear disease can be induced by enlarged and/or inflamed adenoids. A recent study underlines the topographical distribution of biofilm-producing bacteria in adenoid subsites among children with chronic or recurrent middle ear infections: although no statistically significant difference is revealed, this work demonstrates a prevalence of *Staphylococcus aureus* in the nasopharyngeal dome and of *Streptococcus pneumoniae* and *Moraxella catharralis* near the ostium of the ET (Saylam et al., 2010).

1.4 Adenoids and Immunity: a more detailed explanation

Adenoidal immune responses are driven by a network of lymphoid cells, nasal epithelial cells and mucosal epithelial cover that co-operate in order to produce an immunological barrier against **infections** (Figure 5). This could represent the first line of defense against external pathogens, whose action is does not only consist in the physical elimination of microorganisms and large particles via mucociliary clearance, but also in the recognition of pathogens and the activation of primary innate immune response and a consequent adaptive immune response, characterized by the proliferation and differentiation of specific clones in the lympho-epithelial organs of Waldeyer's ring (Ramanathan M Jr et al, 2007).

In particular, several studies revealed that innate immune system has a central role in the protection of mucosal surfaces of adenoids against external pathogens, that are detected by pattern recognition receptors, including TLRs (Toll-Like Receptors) acting as signaling molecules (Ooi EH et al, 2008). Their recognition by TLRs promotes the activation of specific genes that encode pro-inflammatory cytokines, such as as tumor necrosis factor-alpha (TNF-α), interleukin (IL)- 1β, IL-6 and IL-8, antimicrobial peptides (PMPs), and other defense molecules (Kovalchuk LV et al, 2011). It seems that pro-inflammatory cytokines may play a role in the exacerbation of inflammatory processes, that could lead to to chronic otitis, adenoiditis and adenoid hypertrophy (Zielnik-Jurkiewicz B et al, 2016).

Gankovskaya and colleagues investigate gene expression of some TLRs and alpha defensins in children affected by adenoid hypertrophy. They demonstrated that TLR2 and TLR4 gene expression is up-regulated, along with a down-regulation of TLR9 and defensins genes in nasal mucosa of these patients, showing some imbalance in the defense mechanisms at nasal mucosa level. The up-regulation of TLR2 and TLR4 also leads to the production of pro-inflammatory

cytokines as TNF-α, IL1β and IL8. The different TLRs gene expression allows the speculation that innate immunity plays a role in the pathogenesis of hypertrophy of the adenoids. In particular, it seems that binding of TLRs to infectious agents could not only activate nasal mucosal cells, but also stimulate the production of important effector molecules including pro-inflammatory cytokines, together with a reduction of antimicrobial peptides expression and this may be a cause of otitis media and adenoiditis (Gankovskaya LV et al, 2018).

The activity of adenoids as immune-competent organ depends also on the generation of diverse B cells and the secretion of mucosal antibody. Their maturation and proliferation in the germinal centers of secondary lymphoid organs seem to be dependent by specialized CD4 T cells known as follicular T helper cells (TFH), which are particularly abundant both in adenoids and tonsils (Wang H et al, 2014).

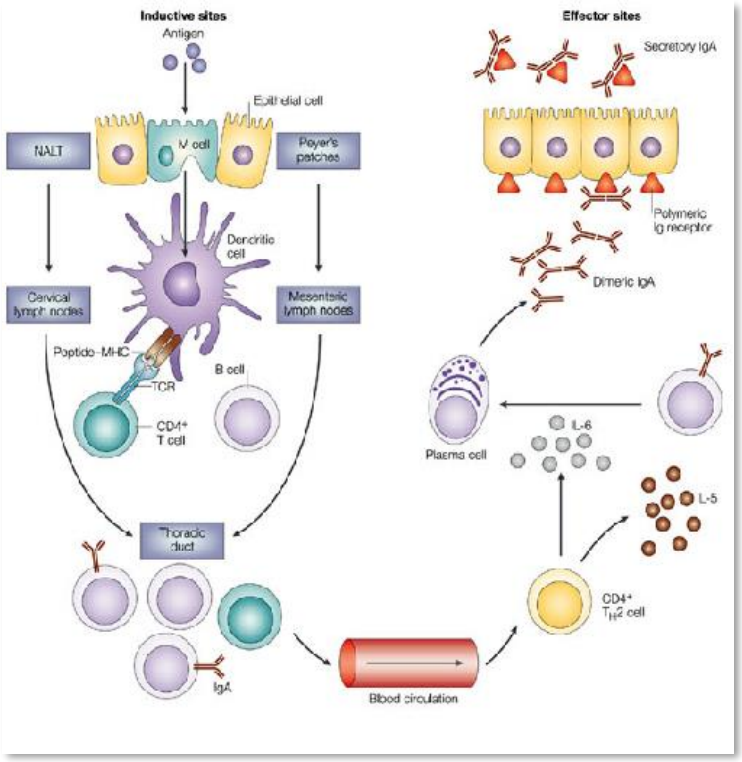

Figure 5: Adenoidal immune-activity

TFH represent a subset of CD4+ T cells expressing a handful of characteristic markers, such as CXCR5 and programmed death 1 (PD-1). CXCR5 is a chemotactic receptor that drives TFH localization to the B-cell zones of lymphoid follicles, while PD-is described as a suppressor of

immune function, in particular of T-cell activity, but it seems also to support the activity of B cells. Another canonical markers for TFH is inducible costimulator (ICOS), that acts as co-stimulatory molecule and promotes the expression of Bcl-6 and induces interleukin 21 (IL-21) production by TFH cells (Crotty S, 2011). This T cell subset is able to support B cell activity by both cell–cell interactions and the secretion of cytokines, such as IL-21 (Berglund LJ et al, 2013).

Morris and colleagues compared T and B-cell function in the tonsils and adenoids by analyzing their response to Staphylococcus enterotoxin B (SEB). They observed that upon polyclonal stimulation with SEB, both TFH and B cells from the adenoids proliferated to a greater extent. This could have important implications also in pediatric surgery because, according to these data, it would be beneficial to delay removal of the adenoids in young children, in order to allow child's immune system to use the greater activity of adenoidal B and T cells (Morris MC et al, 2016).

Moreover, Michea and colleagues demonstrated the presence of plasmacytoid DCs (pDCs) in the perifollicular T-cell, where they are able to effectively drive a competent immune response against *N. meningitides*, releasing TNF-α, IL-6, and IFN-α. It seems that these pDCs, also identified as BDCA-2+ cells, are located in the stratified and pseudostratified epithelium, in direct contact with the microbiota of the upper respiratory tract (Wang H et al, 2014). It seems that pDCs are conditioned by tonsillar epithelial cells receiving signals from the local microenvironment to become less inflammatory in response to the microbiota. This results in a reduction in the release of inflammatory mediators, maintaining a good ability to drive T-cell differentiation, without any influence on T-cell-dependent cytokine production. In this way, pDCs are still capable of initiating adaptive immunity to the external microbes (Michea P et al, 2013; Rescigno M et al, 2013).

Adenoid hypertrophy (AH) is often associated with pediatric chronic rhinosinusitis (pCRS), but its role in the inflammatory process of pCRS is still unclear. In order to detect the expression of innate immunity genes in epithelial cells of hypertrophic adenoids, including antimicrobial peptides and pattern recognition receptors (PRRs), nine pediatric patients affected by pCRS an nine simple AH patients undergoing adenoidectomy were enrolled. After isolation of adenoidal epithelium the relative expression levels of the mRNAs of human β-defensin (HBD) 2 and 3, surfactant protein (SP)-A and D, toll-like receptors 1–10, nucleotide-binding oligomerization domain (NOD)-like receptors NOD 1, NOD 2, and NACHT, LRR and PYD domains-containing protein 3, retinoic acid-induced gene 1, melanoma differentiation-associated gene 5, and nuclear factor-κB (NF-κB) was detected. While the expression of PRRs and NF-κB, as well as HBD-2, HBD-3, and SP-A showed no statistically significant differences between the two

groups, a significant down regulation of SP-D levels was observed in adenoidal epithelium of pCRS patients.

These results suggest that although PRRs are an integral part of the innate immune system, they do not directly contribute to the inflammatory process in pCRS. On the contrary, the collectins SP-A and SP-D are innate immunity proteins secreted from epithelial surfaces. They have an important role in the induction of clearance of pathogens by APCs (antigen presenting cells) and their decreased expression of important in adenoidal epithelium may pave the way for the develpoment of a the inflammatory process of pCRS (Qu XP et al, 2015).

Adenoidal immune response is also regulated by the action of specific antimicrobial peptides called defensis produced in different region of the human body, including oral cavity, and belonging to innate immunity system. β-defensin 1 (hBD-1) is one of the better studied and characterized and its production could be induced in the oral cavity by inflammation (Abiko Y et al, 2007). Zupin ad colleagues analyzed functional polymorphisms within DEFB1 gene, encoding the human beta defensin-1 (hBD-1). They evaluated possible associations between DEFB1 rare haplotypes and susceptibility to adeno-tonsillar hypertrophy in a cohort of pediatric patients with adeno-tonsillar hypertrophy subjected to adeno-tonsillectomy. They observed the expression of DEFB1 mRNA in the tonsils, while the hBD-1 protein was mainly localized at the epithelial level, closer to the basal lamina leading to the speculation that hBD-1 is involved in innate immune response and in the modulation of the susceptibility towards adeno-tonsillar hypertrophy development (Zupin L et al, 2018).

Adenoidal and tonsillar immune responses must be tightly regulated in order to balance the protection against virulent germs and the tolerance to harmless flora and innocuous antigens in the oropharyngeal tract. The regulation of adenoidal immune responses involves also a newly identified interleukin-10 (IL-10)–producing B cell subpopulation, defined as regulatory B cells (Bregs) by Mizoguchi and colleagues (Mizoguchi A et al, 2002).

These cells, that can suppress inflammatory responses in experimental model of autoimmune diseases such as autoimmune encephalomyelitis and collagen-induced arthritis, are implicated in the generation and maintenance of T-regulatory (Treg) cells in the periphery (Mauri C et al, 2012).

The presence of Bregs has been demonstrated in mice and in humans are down regulated in several autoimmune diseases, but their morphological and functional characteristics in human adenoids and tonsils are not clearly identified. As well as in mice, the presence of Bregs has been describe in humans in peripheral blood or in secondary lymphoid organs such as spleen and exclusively in autoimmune disorders. So far little is known about the involvement of B10 cells in

the pathogenesis of chronic inflammatory processes or allergic diseases, while their presence and their phenotypic and functional characterization in adenoids is still controversial (Kalampokis I et al, 2013).

Recently, our group demonstrated for the first time in humans, the presence of a specific subpopulation of IL10 producing Breg cells in adenoids in a cohort of allergic and not allergic children with adenoidal hypertrophy. We showed that the percentage of B10 cells was higher in atopic children, compared to not atopic ones, while no differences was observed in the surface markers CD1d^{hi}, CD5, CD24^{hi} between the two groups. These findings lead us to speculate that the presence of CD19/IL10+ B cells in adenoids of these patients could be probably a consequence of infectious inflammation and atopy could have a role in their expansion (Valsecchi C et al, 2018).

1.4.1 Adenoids and allergy

The most evident correlation is the involvement of adenoids in the development of allergic inflammatory reactions in the upper respiratory airways, even if in literature there is no a great number of studies in this field.

Recently it has been demonstrated that the association between adenoid hypertrophy (AH) and mold exposure in children living in damp environment. In particular, it seems that there is no correlation between adenoid hypertrophy and atopic nature of patients, even if these patients are more prone to the influence of environmental triggers and genetic factors. Vitamin D levels could have a role in the sensitization process. A higher vitamin D level is associated with a lower sensitization risk, while children with recurrent respiratory tract infections grow, they tend to have lower vitamin D levels, become more atopic and tend to have adenoid hypertrophy (Atan Sahin O et al, 2016). According to their results, children with mold exposure had significantly increased adenoid hypertrophy regardless of their atopic nature. As children with recurrent respiratory tract infections grow, they tend to have lower vitamin D levels, a greater possibility to become atopic and to develop adenoid hypertrophy.

Several researchers underlined the possible correlation between AH and allergic rhinitis (AR). Dogru and colleagues found a higher frequency of AR persistency in patients with AH. In particular, they observed that the presence of AH was related an increased severity of AR and prolongs disease duration. On the other hand, there was a negative relationship between AH and asthma in children with AR, so that they suggest to consider and investigate AH particularly in non-asthmatic children with pronounced nasal congestion (Dogru M et al, 2017).

They also previously demonstrated that AH frequency was higher in children with allergic disease compared to controls, and the most common sensitivity to allergens among patients with AH was to house dust. The researchers concluded that the presence of allergic rhinitis and cigarette smoke exposure could be risk factors for developing AH (Evcimik MF et al, 2015).

These results could be particularly important in order to understand the effects of atopy or second hand tobacco smoke (SHS) on the functionality of adenoids in children. It is well known that SHS could have an immune-suppressive effect, with a strongly reduced Th1 and increased Th2 immune response, especially when exposure occurs during fetal development (M. Yu et al, 2008).

Our group previously analyzed the reactivity of adenoidal B cells of pediatric patients undergoing adenoidectomy due to adenoidal hypertrophy. In these patients, adenoidal B lymphocytes have a different reactivity to *in vitro* stimulation with bacterial antigens LPS (Lipopolysaccharide) and CpG and this difference could be related both to exposure to passive smoke and/or atopic condition. SHS is able to influence both innate immune cells, impairing dendritic cell function and NK cell cytotoxicity, and acquired immunity, modulating T lymphocyte activity (Mian MF et al, 2008). In our study, atopy seems to affect *in vitro* response to bacterial antigens, while an increasing trend for IgA and IgM T-independent production associated was related with the presence of both risk factors, reinforcing the hypothesis that the adenoidal inflammatory microenvironment of both exposed and atopic children leads to an exaggerated B cell local response (Tagliacarne et al, 2015).

Moreover, it is well known that atopy, could be defined as a genetic predisposition to develop allergic disorders and is characterized by a defect in T regulatory cells and a Th2-polarized immune response (Tagliacarne et al, 2015). Chronic exposure to SHS leads to an up-regulation of pro-inflammatory pathways with a consequent increase in the levels of some circulating pro-inflammatory cytokines, such as IL1-β, IL6, TNF-α, maintaining a persistent inflammatory state (P.G. Holt et al, 1977).

According to our results, we speculated that SHS does not have direct effects on adenoidal tissue or on local immune cells, but it might determine an inflammatory microenvironment leading to the persistent activation of antibody-producing cells. Even if the role of atopy and allergens in adenoidal hypertrophy is not fully clear, there is however evidence that adenoidal tissue of allergic children is involved in respiratory inflammatory processes (Tagliacarne et al, 2015).

Previously, Alexopouls and colleagues analyzed the possible correlation between atopy and the frequency of adenotonsillar hypertrophy, that is one of the clinical factors associated with

increased upper airway resistance and, or, pharyngeal collapsibility that predispose to obstructive sleep apnoea (OSA). On the contrary, the role of atopy in the in the pathogenesis of adenotonsillar hypertrophy and obstructive sleep apnoea (OSA) is still controversial (Alexopoulos EI et al, 2014).

Also allergic rhinitis has a negative impact on sleep quality in children, but it is unclear whether OSA is one of the pathophysiologic mechanisms that mediate this adverse effect (Koinis-Mitchell D et al, 2012). They postulated that the contribution of allergic rhinitis to the pathogenesis of sleep-disordered breathing can be quantified more accurately in these children in whom there is a clinical indication for adenotonsillectomy to treat OSA. According to this study, adenotonsillar hypertrophy seems to be the predominant risk factor for OSA in childhood when compared to atopic predisposition (Alexopoulos EI et al, 2014).

One of the primary cause of sleep respiratory disorders (SRDs) in children is represented by adenotonsillar hyperplasia (AH). It has been reported that cysteinyl leukotrienes (CysLTs) and their receptors Cysteinyl leukotriene receptor 1 and 2 (CysLTR1 and CysLTR2) have a role in AH pathogenesis, through the stimulation of lymphocyte proliferation in tonsils (Tsaoussoglou M et al, 2012). The use of antileukotrienes drugs is reported to be effective also on allergic patients, even if the relationship between allergic rhinitis and AH has not been completely demonstrated (Kuhle S et al, 2011). Paulucci and colleagues compared the expression of CysLTR1 and CysLTR2 in the B and T cells of hyperplasic tonsils of sensitized (SE) and control (NS) snoring children.

Mononuclear cells from surgical removed adenoids and tonsils were stained for CysLTR1, CysLTR2, CD19 for B lymphocytes, and CD3 for T lymphocytes and counted via flow cytometry, while the expression of the CysLTRs genes was measured real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). CysLTR1 and CysLTR2 expression was higher in the lymphocytes of hyperplasic tonsils in non allergic children both by CD3 and CD19 lymphocytes and both SE and NS cells had higher expression of CysLTR2 than CysLTR1. Considering previous findings (Kuhle S et al, 2011), the researchers suggested that that both receptors could be involved in cellular proliferation and hyperplasia. However, but the development of new drugs capable of blocking both CysLTR1 and CysLTR2 could have a positive impact in the nonsurgical treatment of AH (Paulucci BP et al, 2016).

During inflammatory processes associated with allergic reactions various inflammatory cells such as neutrophils, basophils and above all eosinophils, infiltrate the inflamed tissue and lead to clinical manifestations in late phase reactions. The cells involved release several types of cytokines and chemokines such as IL-4, IL-5, IL-13, Vascular Cell Adhesion Molecule-1 [VCAM-I], Intercellular adhesion molecule-1 [ICAM-1]), that is one of the mechanisms regulating the migration of specific eosinophils from the circulation to allergic inflammatory sites. Allergic

inflammation in nasal and bronchial mucosa, but also in adenoids and tonsils, is characterized by a marked infiltration of eosinophils in the tissue (R. Pawankar et al, 2000; M.S. Dykewicz et al, 2010).

In order to analyze the possible association between adenotonsillar tissue eosinophilia and allergy, a Turkish research group performed hematoxylin-eosin stained sections of adenoidal and tonsillar samples after surgical removal. The allergic sensitization of patients was demonstrated after skin prick test and the patients were also investigated for allergic rhinitis. This study demonstrated that the number of eosinophils were significantly higher in tissue samples from allergen sensitized patients. In particular, the researchers concluded that if number of eosinophils resulted ≥5/10 high-powered fields in adenoid tissue and ≥3/10 high powered fields in tonsil tissue represented, the child can be accepted as sensitized. This is interesting above all because, since usually allergic rhinitis and adenotonsillar disease seem to be interrelated, the examination of specimens of the same sample in terms of eosinophilia may facilitate the diagnosis and the treatment of allergy at the postoperative follow up of patients (Ekici NY et al, 2018).

1.4.2 Adenoids and passive smoke

Passive exposure of children to cigarette smoke is implicated in several childhood respiratory disorders. It has been demonstrated that IFN-g production by CD8 Tcells is defective in these children, predisposing to recurrent respiratory infections (Marseglia et al., 2009). Moreover, as highlighted by many authors, passive cigarette smoking increases proinflammatory molecules, deescalates the Th1/Th2 ratio and can induce several structural changes in the respiratory nasal mucosa with negative consequences for its ciliary activity and mucociliary function (Elwany et al., 2012).

Because of these effects, the defense mechanisms of the nose may be vitiated or lost, and those children can develop persistent adenotonsillar and respiratory infections (rhinitis, sinusitis, pharyngitis, otitis, laryngitis, bronchitis, and pneumonia) and allergic diseases. Children are more significantly affected by passive smoking than adults, because their respiratory and immune systems are not fully developed.

In particular, it has been previously observed in pediatric subjects that passive exposure to tobacco smoke strongly reduced the percentage of IFN-γ producing CD8+ T cells, enhancing the susceptibility to recurrent of respiratory infections (Ruskamp et al, 2010). Moreover, TLR expression on cytotoxic T cells could be affected in children by second-hand tobacco smoke (Holt PG et al, 1977). Lannero and colleagues demonstrated that exposure to tobacco smoke in early infancy may be associated with an increased risk of atopic sensitization and respiratory tract infections (W. Jiang et al 2007; V. Hornung et al, 2002). About the inflammatory effects of passive

smoke exposure on the adenoidal microenvironment and immune cells, our previous data showed an increase in IgA, IgG and IgM production in an in vitro T-independent system in children exposed to smoke, while no differences were observed after TLR stimulation. These results indicate that passive exposure does not seem to affect B cell function and cellular antigen-presentation ability in the adenoidal tissue. This could explain why we found an increase of in vitro immunoglobulin production in exposed children. Chronic exposure to tobacco smoke results in an up-regulation of pro-inflammatory pathways and an increase in the levels of some circulating cytokines, such as IL1, IL6, TNF, maintaining a persistent inflammatory state (Matt et al, 2000). We speculate that second-hand tobacco smoke does not have direct effects on adenoidal tissue or on local immune cells, but it might determine an inflammatory microenvironment leading to the persistent activation of antibody-producing cells. Our data suggest that B cells are more sensitive to exter-nal antigens in this context, this could explain the increase in immunoglobulin production in the T-independent system. Also TLR dependent immunoglobulin production was influenced by this exposure, but the differences between exposed and non-exposed patients were less evident and, consequently, not statistically sig-nificant. Moreover, it is known that TLR9 is primarily involved in recogni-tion of CpG motifs in B cells and plasmacytoid dendritic cells (PDC) (Tagliacarne et al 2015). Although previous studies showed that naïve B cells can proliferate in direct response to CpG ODN and produce IgM (Koinis-Mitchell D et al, 2012), Hornung et al. demonstrated that in B cells, TLR7 was down-regulated in response to CpG ODN and CpG-induced regulation of TLR7 expression may explain the modulation of the recognition of CpG motifs by TLR9.

1.4.3 Adenoids and inflammation

Several different external stimuli can lead to an imbalance of local adenoidal immune cells and to the development of an abnormal inflammatory response that may lead to delayed healing, causing immune activation in the adenoids and adenoidal hypotrophy. This condition could lead to obstructive sleep apnea (OSA) that in children is usually caused by structural or functional abnormalities or by the obstruction of the upper airway (M.F. Mian et al, 2008).

A research by Ni and colleagues aimed to explore the role of Th17/Treg ratio in adenoids of children with OSA and its possible relationship with allergic rhinitis, assuming that adenoidal immune homeostasis is closely linked to the incidence of OSA and to sleep disorders that could strongly affect the number and functionality of T reg cells (Ganz F.D et al, 2012). In this study, the number of Th17 and Treg cells, the levels of IL-17, IL-10, and TGF-beta in cellular secretions, and the expression of key transcription factors RORγt and Foxp3 in both the peripheral blood and adenoid tissue of children diagnosed with OSA by polysomnography (PSG) was evaluated.

The results showed that OSA children exhibited a significant increase in the number of peripheral Th17 cells, IL-17 secretion, and RORγt mRNA levels, and a concomitant decrease in the number of Treg cells, IL-10 and TGF-β secretion and Foxp3 mRNA levels. Moreover, Th17/Treg ratio was higher ($p < 0.05$) in the OSA groups than in the control group and was correlated with the size of the adenoids. According to their results, seems that there was a direct correlation between Th17/ Treg balance in OSA patients and the complication with allergic rhinitis, as observed both in the peripheral blood and local adenoid tissue. The researchers concluded that an imbalance in the Th17/Treg ratio could facilitate the risk of developing OSA, while AR may promote the development of the disease, providing an alternative explanation for OSA pathogenesis and giving new directions for the treatment and prevention of this disease in children (Ni K et al, 2015).

The regulation of immune responses against pathogens in the oral cavity is mediated primary by the innate immunity, while adenoids and tonsils are able to provide a fast and unspecific response to pathogens as well as to trigger the action of the acquired immune system, due to the constant contact with the external milieu and extraneous materials (L. Brodsky et al, 1988). Moreover, it is known that chronic rhinosinusitis (CRS) and asthma frequently coexist both in children and adults and the epidemiologic link between these two diseases has been confirmed by pathophysiologic and therapeutic observations. In particular, histologic studies have shown the presence of mast cells and eosinophils both in the nasal mucosa of subjects with allergic rhinitis and in the bronchial mucosa of asthmatic patients. However, the precise interaction between asthma and CRS is still poorly understood, especially in children (Dejima K et al, 2005). This is due primarily to the lack of direct measurements of mucosal inflammation comparing the upper airways of non-asthmatic and asthmatic children, as most patients are managed medically and do not require surgery. Anfuso and colleagues analyzed the expression of a large array of inflammatory cytokines and chemokines in the sinus and adenoid tissues surgically removed from pediatric subjects with CRS refractory to medical management, in comparison with control subjects without upper or lower airway disease. Children with CRS and asthma had significantly higher sinus levels of tumor necrosis factor-α and adenoid levels of epidermal growth factor, eotaxin, fibroblast growth factor-2, growth-related oncogene, and platelet-derived growth factor-AA, compared with children with CRS but without asthma (Anfuso et al, 2015).

These results showed that the inflammatory response in the upper airway mucosa of children with asthma and CRS was more severe, compared with children with CRS without asthma. This lead the researchers to speculate that asthma in these patients is caused or exacerbated by severe upper airway disease and speculate that the treatment of treating sinus and adenoidal diseases could be for the management of chronic asthma in children.

It has been also demonstrated that specific pathogens, such as *Chlamydia pneumoniae,* have a role in the infective process in adenoid pathology, even if the number of studies are published in this field due to difficulties in sustaining bacterial cultures and to low accessibility to other methods that could confirm infection, as PCR and/or immunohistochemistry. In a cohort of children children aged from 2 to 16 years (mean age 6,4) undergoing adenoidectomy, real-time PCR for *C. pneumoniae* was performed in adenoid tissue after surgical removal. Adenoids from children with positive PCR examination and from 10 children with negative PCR examination were examined using immunohistochemistry (IHC). In this study, a correlation between *C. pneumoniae* occurrence in an adenoid tissue and the size of adenoid was observed, while the presence of the pathogen was detected primary in adenoidal lymphocytes and in epithelial cells.

These result confirm the involvement of *C. pneumoniae* in the pathogenesis of adenoid pathology, contributing to hypertrophy or chronic inflammation (Bielicka A et al, 2016).

In airway epithelium, local inflammation leads to a modification of mucus viscosity, especially as a consequence of pro-inflammatory cytokines production, such as TNF-α. An *in vitro* study analyzed the effect of TNF-α stimulation on autoregulatory mechanisms that regulates ciliary beat frequency (CBF) on ciliated cells cultured from human pediatric epithelial adenoid tissue. Using dextran solutions to increase viscosity, the treatment with TNF-α produced a significantly decrease in CBF in cell cultures exposed to dextran solutions. Even if TNF-α could have negative effects on the functionality of ciliated cells in response to maintaining CBF after increasing viscosity, it could prevent MCC collapse through increasing ntracellular Ca+2 level. Concluding, it seems that the increase of TNF-α levels in nasopharyngeal epithelium during inflammation may contribute to the maintenance of effectiveness of MCC in upper airways (González C et al, 2016).

In many cases it has been documented that clinical complications of adenoid vegetation, hypertrophic palatine tonsils and recurrent tonsillitis that lead to either tonsillectomy or adenotonsillectomy. Even if the pathophysiology of these diseases remains largely unknown, it has been demonstrated that Activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA-editing catalytic polypeptide 3 (A3) family could have a role in B-cell maturation, carcinogenesis and also in antiviral immunity (Knisvacher, BA et al, 2016).

Finally, analyzing the expression profile of AID and A3s in adenoids and palatine tonsils of adult patients, a recent study observed that AID was expressed independently from A3s in the adenoids, but AID and A3s were correlated with each other in the adenoids and the palatine tonsils. Moreover, immunohistochemical experiments showed that there was a negative correlation between age and AID or A3 expression in the adenoids and a positive correlation in

palatine tonsils, although no statistically significant difference between the palatine tonsils with hypertrophy and those with repeated inflammation was evaluable. The researchers also found a higher AID and A3s expression in the epithelial cells of the adenoids and the palatine tonsils, compared with that in germinal centers (GCs). Due to the fact that aberrant AID or A3s expression is associated with inflammation, it could be hypothesized that the localization of AID expression and its correlation with age of patients may contribute to adenoid vegetation and inflammation that predict the need of their surgical removal (Seishima N et al, 2018).

1.5 Diagnosis of adenoid disorders

Various methods for visualizing adenoids have been reported in the past such as posterior rhinoscopy (with laryngeal mirror) and nasopharyngeal radiological examination. Flexible fiber-optic nasal endoscopy is nowadays an established and widely used method in routine ENT practice as it permits the regions of interest to be visualized and enables a correct diagnosis to be made. As previously stated, the main cause of nasal obstruction in children is adenoid hypertrophy; but other causes should be considered such as allergic rhinitis, rhinosinusitis, nasal polyposis, septal deviation, choanal atresia, and sinonasal tumors. These rare conditions can remain undiagnosed if a nasal endoscopy is not performed.

1.5.1 Nasal endoscopy

The introduction of flexible optic scopes into ENT made it possible to inspect the nasal cavities, including those in children (Selner, 1988; Kubba and Bingham, 2001). Rigid endoscopy is considered the "gold standard" for nasal examination in adults as it provides a good quality of image, but it can be difficult to perform on nonsedated children. In contrast, the flexible fiber-optic scope is a safe instrument. Some authors consider nasal flexible fiber-optic endoscopy as the method of choice for evaluating the nasopharynx in children (Cassano et al., 2003; Chien et al., 2005). Endoscopic examination allows the nasal cavities and nasopharynx to be observed directly and permits a more accurate diagnosis to be made. Nevertheless, nasal endoscopy can present some disadvantages: as some authors state, it allows little opportunity for objective measurement, often causing low interobserver agreement (Filho et al., 2001; Major et al., 2006). Studies by Lertsburapa et al. (2010) on 99 children revealed a correlation between flexible fiber-optic nasal endoscopy and intraoperative nasopharyngoscopy performed during adenoidectomy. In this study, flexible nasal endoscopy correlated highly with the standard intraoperative nasopharyngoscopy (Lertsburapa et al., 2010). Various methods for grading adenoid hypertrophy have been reported in the literature (Cassano et al., 2003; Josephson et al., 2011). The classification proposed by Parikh et al. (2006) is effective for evaluating the degree of obstruction of adenoid

tissue over the posterior choanae in the nasopharynx: grade 1 for adenoid tissue not in contact with adjacent structures; grade 2 for adenoid tissue in contact with the torus tubarius; grade 3 for adenoid tissue in contact with the vomer; and grade 4 for adenoid tissue in contact with the soft palate (at rest; Parikh et al., 2006). A prospective study of 180 pediatric patients confirmed that the size of the adenoids assessed during nasal endoscopy correlated very well with nasal obstruction, and that the condition of the nasopharyngeal orifice of the ET significantly corresponded with the type of tympanogram. The authors also reported that nasal endoscopy gave accurate information for indications of adenoidectomy. Moreover, this examination is possible in most cases if performed by a skilled otorhinolaryngologist and preceded by careful explanation to the child. Finally, the possibility of direct visualization of the image via a monitor allows the clinical situation to be explained better to the child's parents (Wang et al., 2012). The authors later confirmed their 1997 results on a larger cohort of 817 children (Wang et al., 1997). Studies by Kindermann et al. (2008) on 133 children revealed the high sensitivity and specificity of nasal flexible fiber-optic endoscopy in the diagnosis of adenoid hypertrophy. In this study, nasal obstruction symptoms such as noisy breathing, snoring and history of sleep apnea correlated significantly with adenoid hypertrophy. According to these authors, nasal endoscopy is a highly accurate, safe, dynamic, and objective diagnostic method and is easy to perform on cooperative children (Kindermann et al., 2008). A prospective study by Yilmaz et al. (2008) revealed that nasal endoscopy is the best way to evaluate a candidate for adenoidectomy, and mirror examination, palpation and volume of tissue removed during surgery correlated well with nasal endoscopy findings (Yilmaz et al., 2008). In 2011, we reported our personal 11-year experience (1999–2010) with nasal endoscopy performed on 6036 pediatric patients, the largest series reported in the literature. In our experience, nasal endoscopy is a reliable, safe, accurate, easily tolerated, and dynamic diagnostic method for assessing adenoid size in children if the correct endoscopes are used under appropriate conditions (Pagella et al., 2011b). A need for anesthesia (topical and general) or vasoconstriction of the nasal mucosa has been reported in the literature, especially for younger children because of their poor cooperation (Wang et al.,1995). However, in our opinion, a careful explanation to the child and a skilled operator can ensure successful nasal endoscopy in most cases.

1.5.2 Radiological examinations

Lateral radiological evaluation of the nasopharynx was performed on children for many years to evaluate adenoid size. Some authors have reported that radiographs are as important as clinical examinations (Haapaniemi, 1995). Radiographs were chosen as the gold standard by Paradise et al. because they correlated well with the volume of adenoid tissue removed during surgery.

Moreover, they are objective and noninvasive tools for estimating the extent of the adenoid tissue inside the nasopharynx (Paradise et al., 1998). However, it is often difficult to carry out this examination in infants, and in many cases, there is no direct correlation between the level of obstruction evidenced by X-ray and nasal symptoms (Cassano et al., 2003). Furthermore, the risk of exposing children to radiation should not be ignored (Cho et al., 1999). Many methods of interpreting adenoid size using radiographs have been described. The most common is the adenoid-to-nasopharyngeal (A/N) ratio, described by Fujioka et al. (1979). This method consists in determining the ratio between the measurement of the adenoid tissue (defined by the distance between the basiocciput region and the most convex part of the adenoid pad) and the nasopharyngeal aperture (defined by the distance between the sphenobasiocciput and the posterior edge of the hard palate; Fujioka et al., 1979; Cohen and Konak, 1985).

1.6 Surgery: adenoidectomy techniques

Adenoidectomy is a very common surgical procedure performed by otolaryngologists. The most common indications for adenoid surgery in patients with adenoid hypertrophy are nasal obstruction, sleep apnea, OM with effusion, and recurrent OM. Techniques and instruments have changed over the years: the standard transoral adenoidectomy technique is performed with an adenoid curette or an adenotome under general anesthesia via oro-tracheal intubation (Kornblut, 1987; Paradise, 1996). The procedure is usually performed without direct visualization of the nasopharynx. The surgical field can be partially visualized by retracting the soft palate with rubber catheters or with a laryngeal mirror: this is particularly useful for avoiding injury to important structures such as the ET and pharyngeal muscles, reducing the risk of postoperative complications. As reported in the literature, conventional curettage adenoidectomy does not achieve adequate removal of adenoid tissue in one-third of children, especially when there is an intranasal, superior or peritubaric extension (Pagella et al., 1996; Buchinsky et al., 2000; Stanislaw et al., 2000; Elluru et al., 2002; Havas and Lowinger, 2002; Murray et al., 2002). To reduce the likelihood of morbidity and to prevent recurrences, total excision of the adenoids is an important goal of this surgery. Digital palpation of the nasopharynx performed at the end of the surgical procedure is still a valid method and is currently used by some otolaryngologists (Buchinsky et al., 2000). However, angled mirror or an endoscope provides adequate visualization of the field during the adenoidectomy, and these techniques are nowadays preferred (Brodsky, 1996; Cannon et al., 1999; Discolo et al., 2001; Ezzat, 2010). The quality of the endoscopic image is unquestionably better than the image obtained using a laryngeal mirror. The endoscopic control can be either transoral (with 70_-angled scope) or transnasal (with 0_ scope). Under the endoscopic control a finer and more assured peritubaric and perichoanal tissue clearance is possible, and the depth of

tissue resection is better controlled. Moreover, tissue is removed carefully with concomitant visual protection of important nearby structures. In recent years, different surgical techniques have been proposed to ensure a finer and more assured removal of adenoids. Suction diathermy was initially introduced for hemorrhage control following conventional curettage (Kwok and Hawke, 1987); subsequently, the whole procedure was performed with this technique (Sherman, 1982; Shin and Hartnick, 2003; Owens et al., 2005; Skilbeck et al., 2007). The introduction of microdebriders in endoscopic sinus surgery (ESS) prompted the use of a shaver system in adenoidectomy; however, the approach was still transoral with indirect visualization of the nasopharynx through a laryngeal mirror (Koltai et al., 1997, 2002; Heras and Koltai, 1998; Stanislaw et al., 2000; Murray et al., 2002; Rodriguez et al., 2002). The advent of ESS popularized the use of intranasal scopes and endoscopic adenoidectomy became the natural evolutionary successor of conventional adenoidectomy, permitting the surgical field to be visualized directly (Becker et al., 1992; Cannon et al., 1999). With this technique, adenoids located along the superior portion of the nasopharynx, the choanae and the peritubaric region can be clearly visualized and thus removed. In subsequent years, a report by Parson (1996) and studies by Yanagisawa and Weaver (1997) revealed power-assisted adenoidectomy conducted entirely through a transnasal approach and under an endoscopic (Parsons, 1996; Yanagisawa and Weaver, 1997). There are several published reports about the use of power-assisted instruments in adenoid surgery. Such techniques can be schematically divided into nonendoscopic, usually performed with a laryngeal or a dental mirror (Koltai et al., 1997; Heras and Koltai, 1998; Stanislaw et al., 2000; Koltai et al., 2002; Murray et al., 2002; Rodriguez et al., 2002), and endoscopic-assisted (Pagella et al., 1996, 2010; Havas and Lowinger, 2002; Costantini et al., 2008; Al- Mazrou et al., 2009). Endoscopic control can be obtained either transnasally or transorally, and the microderbider can be inserted into the nasal cavity (straight blades) or maneuvered through the oral cavity (curved blades). Some authors report the use of power-assisted instruments throughout the adenoidectomy; according to others, the microdebrider can be used as a step in the surgical procedure (combined techniques). Studies by Havas and Lowinger (2002) revealed combined power-assisted techniques (Havas and Lowinger, 2002), which were then reproposed by Pagella et al. (2009; Pagella et al., 1996). As a first step in these techniques, a traditional transoral adenoidectomy with adenotome and curette was performed; then, if persistent residual adenoid tissue still obstructed the nasopharynx significantly, the adenoidectomy was completed using a transnasal powered shaver under a transnasal endoscopic view. The cutting and aspirating action of the shaver removed both adenoid tissue and blood, providing a clear surgical field and keeping the tip of the instrument under visual control. Use of the transnasal microdebrider and transnasal endoscopic view ensures complete adenoidectomy and, in particular, better control over the extent of resection, especially around

the choanal sill and the torus tubarius (Pagella et al., 1996; Yanagisawa and Weaver, 1997; Havas and Lowinger, 2002; Al-Mazrou et al., 2009). However, this technique could be difficult especially for young children with narrow nasal passages. In such cases, it can be difficult to introduce both instruments into the nasal cavity, and surgical maneuverability is also limited by the contact between the hand-piece and the scope attachment. Following these developments, studies by Pagella et al. (2010) revealed the transoral endonasalcontrolled combined adenoidectomy (TECCA; Pagella et al., 1996). In this technique, if residual adenoid tissue still obstructs the nasopharynx after a traditional adenoidectomy, the patient undergoes completion of the adenoidectomy with a curved 60_ transoral microdebrider under endoscopic transnasal control. Implementation of this technique seems to have addressed some problems encountered during the latter procedure. Both procedures are similar in safety and efficacy. The TECCA technique permits better maneuverability of the instruments if the nasal spaces are narrow. Moreover, this method can be effective in removing lateral (peritubaric) and superior adenoid tissue with excellent precision (Pagella et al., 2011a). In conclusion, there are several reports about the usage of power-assisted instruments in adenoid surgery. The visual control can be endoscopic or non-endoscopic, and the shaver can be used transorally or transnasally. All recent experiences emphasize the advantages of endoscopic techniques in performing adenoidectomy. These techniques are safe and effective, and the personal experience of the surgeon should guide the choice of the instruments and of the approaches.

2 STUDY

2.1 Aim of the study

The main purpose of this study is to evaluate potential changes of adenoidal immunity in response to atopy, exposure to passive smoke and low dose medicine compound in a cohort of children with adenoid hypertrophy, requiring adenoidectomy and/or adenotonsillectomy.

Moreover, this research is intended to emphasise the possible correlation between the immunosuppression induced by atopy or passive smoking and the immunomodulating activity of the low dose medicine compound.

PART I

2.2 Materials and Methods

2.2.1 Patients

A total of 277 children (151 males and 126 females; median age 5.5 years), with adenoidal hypertrophy requiring adenoidectomy and or adeno-tonsillectomy, were consecutively enrolled in the study. All patients were evaluated by an ear nose throat (ENT) specialist, who performed fiberoptic nasal endoscopy to confirm the diagnosis of adenoidal hypertrophy with an indication for surgery to treat upper airway obstruction. A pediatrician examined each patient and collected a complete medical history. Subjects were initially subdivided into two groups according to the presence of atopy: atopic (n = 97) and non-atopic (n = 168) children. Atopy was defined as the presence of one (or more) relative who was affected by an allergic disease, according to validated criteria [20]. We performed prick tests as allergy screening for all patients. Not for all atopic and/or not atopic patients after smoke exposure was possible to perform IgE production. Parents were also asked whether their child was in close contact with any active smoker (one parents or both, any primary caregiver involving grandparents or, less frequently, nannies and other relatives). In the present study, we classified as "exposed to tobacco smoke" those children in close contact

with at least one active smoker. Therefore, on the basis of these criteria, patients were classified into two subgroups: those who had been exposed (82 children) and those who had not (195 children). Children with an immunodeficiency, chronic syndrome or pathologic obesity were excluded. Children who chronically received systemic corticosteroids were also excluded. The present study was approved by the Ethics Committee of "Fondazione IRCCS Policlinico San Matteo, Pavia", and written informed consent by both parents was obtained before enrollment.

2.2.2 Adenoidal mononuclear cells (AMC) separation

After adenoidectomy, adenoids were pressed through a filter to eliminate stromal tissue and adenoidal mononuclear cells (AMC) were processed as previously described (). Briefly, AMC were isolated after a centrifugation on a Ficoll-Hypaque gradient and resuspended in a freezing solution containing fetal calf serum (FCS, Euroclone) and 10% dimethyl sulphoxyde (DMSO; Edwards Life-sciences) and cryopreserved at −180 ◦C until evaluation (Figure 6).

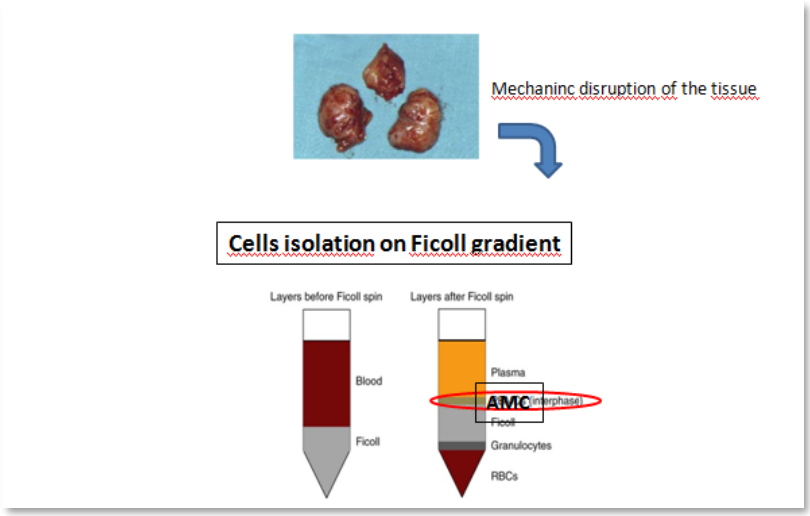

Figure 6: Schematic representation of the AMC separation

2.2.3 Lymphocyte phenotyping and *in vitro* immunoglobulin production

2.2.3.1 *Adenoidal lymphocyte T-independent immunoglobulin production*

AMC were thawed at 37°C and processed for T-independent CD40-system *in vitro* immunoglobulin production as described by Marconi et al. (*Marconi M. et al. 1998*).

Briefly, surface phenotype markers were studied by FACS analysis using fluorescein-conjugated monoclonal antibodies: anti-human CD45 (Beckman Coulter) and anti-human CD19 (Beckman Coulter). Cells were analyzed using a Navios Beckman Coulter flow cytometer.

Next, AMC (10^5/well) were resuspended in Iscove's medium (Gibco) supplemented with 5 g/ml bovine insulin (Sigma), 0.5% bovine albumin (Sigma), Hepes buffer 0.01 M (Sigma), 1 mM glutamine (Gibco), 0.025 mg/ml gentamicin (Gibco), 1g/ml ethanolamine (Sigma).

The CD40 system was performed using an irradiated (70 Gy) CDw32 mouse fibroblast cell line (2×10^3/well) presenting mouse anti-human CD40 (0.5 g/ml, Calbiochem).

This system, was set up to induce B lymphocytes immunoglobulin production, without T-dependent antigen presentation. Purified recombinant human IL-10 (R&D System) was also used at the concentration of 294 ng/ml. All conditions were run in triplicate.

Cells were incubated for 10 days at a final volume of 200 µl/well in round bottom 96 well micro-plates (Corning Costar) at 37°C in a humidified 5% CO2 atmosphere. Culture medium has not been refreshed for 10 days in order to maintain in the supernatants the first immunoglobulin produced.

2.2.3.2 *Modified adenoidal lymphocyte T-independent immunoglobulin production*

To assess the ability of adenoid stromal cells to induce B cell proliferation, activation and immunoglobulin production in the GC, AMC were thawed at 37◦C and processed for T-independent *in vitro* immunoglobulin production using a modified method, as described by Tagliacarne et al., (2015)

Briefly, lymphocyte surface phenotype markers were studied by FACS analysis using fluorescein-conjugated monoclonal antibodies: anti-human CD45 (Beckman Coulter) and anti-human CD19 (Beckman Coulter). Cells were analyzed using a Navios Beckman Coulter flow cytometer.

The induction system was performed using irradiated (70 Gy) ASC from different batches at P3. Irradiated ASC were plated at the final concentration of (2×10^3/well) and incubated 24 hours at 37◦C in a humidified 5% CO2 atmosphere to let them become adherent to the plate.

Next, the following day AMC (10^5/well) were resuspended in Iscove's medium (Gibco) supplemented with 5 g/ml bovine insulin (Sigma), 0.5% bovine albumin (Sigma), Hepes buffer 0.01 M (Sigma), 1 mM glutamine (Gibco), 0.025 mg/ml gentamicin (Gibco), 1g/ml ethanolamine (Sigma).

This system was set up to induce B lymphocytes immunoglobulin production and purified recombinant human IL-10 (R&D System) was also used at the concentration of 294 ng/ml. All conditions were run in triplicate.

Cells were incubated for 10 days at a final volume of 200 µl/well in round bottom 96 well micro-plates (Corning Costar) at 37°C in a humidified 5% CO2 atmosphere. Culture medium has not been refreshed for 10 days in order to maintain in the supernatants the first immunoglobulin produced.

2.2.3.3 *Adenoidal lymphocyte immunoglobulin production after stimulation with TLRs*

For the evaluation of in vitro immunoglobulin production after stimulation with TLR ligands, AMC were re-suspended in RPMI1640 (Euroclone) supplemented with 1% glutamin and 0.1% gentamicin and 10% fetal calf serum (FCS, Euroclone) and 10 ng/ml recombinant human IL2 (Genzyme Diagnostics).

Cells (10^5/well) were then incubated in round bottom 96 well micro-plates (Corning Costar) for 10 days, without refreshing the medium, in the presence of 25 g/ml TLR9 ligand bacterial un methylated CpG oligonucleotides (Roche Diagnostics) and 1 g/ml TLR4 ligand lipopolysaccharide (LPS, Sigma), at 37°C in a humidified 5% CO2atmosphere. Culture supernatants were then harvested and immunoglobulin levels were ELISA assayed.

2.2.4 Quantification of IgA, IgG and IgM levels in culture supernatants

Immunoglobulin levels in culture supernatants were detected using an ELISA technique as described by Avanzini et al. (Avanzini MA. et al. 1992).

Microtiter plates (Greiner) were coated with polyclonal rabbit anti-human IgA, IgG and IgM (Dako Immunoglobulins) and incubated for 3 h at 37°C and then overnight at 4°C.

The second day, culture supernatants were incubated for 2 h at 37°C and then secondary rabbit anti-human IgA, IgG, and IgM antibodies conjugated to horse-radish peroxidase (HRP) (Dako) were added. The plate was read at 492 nm in a micro-plate reader Sunrise (Tecan) and the concentration of each immunoglobulin was extrapolated from a standard curve included in each plate and expressed in ng/ml.

2.2.5 *In vitro* detection of IL10-producing B regulatory (Breg) cells in adenoids

2.2.5.1 *In vitro stimulation with CpG- ODN and CD40L*

AMC were thawed at 37°C and then resuspended at the concentration of $2x10^6$cells/ml and in 12 well flat bottom plates (Corning Costar) in RPMI 1640 medium (Euroclone) + 10% Fetal Bovine Serum (FBS, Euroclone). The stimulation protocol was partially modified by Iwata et al [18]. Briefly AMC were then incubated for 72h with 10 μg/ml CpG ODN 2006 (Roche) and 1μg/ml CD40L (R&D system) for 72h at 37°C, 5%CO_2

2.2.5.2 *Detection of intracellular IL10 production and surface phenotype*

As described by Valsecchi et al., (2018), for the evaluation of intracellular IL10 production, 10 μg/ml Brefeldin A (eBiosciences), 1 μg/ml Ionomycin (Sigma Aldrich) and 50 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) were added for the last 5 hours of culture with CpG-ODN 2006 and CD40L. Cells were then harvested and resuspended in RPMI 1640+10% FBS, then labelled with monoclonal antibodies (mAbs) for surface markers. The following antihuman mAbs were included: anti human CD19 PC7 (Beckman Coulter), CD1d APC (Biolegend), CD5 FITC (Becton Dickinson), CD24 APC (Biolegend). Cells were incubated with mAbs for 20 minutes in ice, then washed with Phosphate-buffered saline (PBS, Euroclone) + 10% pool human serum. Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (eBiosciences) according to the manufacturer's instructions then stained with anti– human IL-10 mAb (Becton Dickinson) for intracellular labelling. After washing with PBS cells were analyzed using Beckman Coulter Navios cytometer.

PART II

2.2.6 *In vitro* stimulation of AMCs with a low-dose immunomodulant product (citomix)

2.2.6.1 *Citomix composition and description*

The medication investigated in this study, is s a low-dose multicomponent medicine, called citomix, which includes components of plant origin (concentration: 10-3 M), components of animal origin (swine organ derivatives; concentration: 10-9 M), and components of biological origin (interleukins, growth factors, and interferons; concentration range: below 10-15 M).

According to Tagliacarne et al 2018, the composition of the product has been studied to activate the innate and cell-mediated immune system limiting, at the same time, the inflammatory process (Figure 7). It is possible to identify three groups of components:

1) The botanical components belong to the phytotherapy tradition. Their concentrations have been carefully chosen in order to modulate the acute inflammatory symptoms:

- *Ananassa sativa,* by its content in bromeline, is traditionally used to treat a wide range of inflammatory conditions (De Lencastre N et al, 2016).
- *Centella asiatica* exerts immuno-modulating and anti-inflammatory activities (Cao W et al, 2010).
- *Vaccinium vitis* is assumed to be a potent antioxidant and anti-inflammatory agent by its flavonoid compounds fraction (Vollmannová A et al, 2009)

2) The biological components contained in citomix are mainly immunity modulators (their concentrations were identified among the most consistent ones for the modulation of the immune system (Castiglioni et al, 2017):

- *G-CSF* is a pleiotropic hemopoietic growth factor that regulates the proliferation and differentiation of progenitor cells within the bone marrow and the release of mature neutrophils into the peripheral blood, increasing ADCC (Antibody Dependent Cell-Mediated Cytotoxicity) and superoxide anion production (EMA, 2014)
- *Interferon-γ* (IFN-γ) exerts a strong immunoregulatory action on several cells driving the Th0 lymphocytes differentiation to Th1 inducing the maturation of CD8+, macrophages activation and NK cytotoxicity. It is crucial for immunity against intracellular pathogens and exerts potent phagocyte-activating effect (R. Pawankar et al, 2000)
- *IL-1β* is normally produced in response to infection, injury, or immunologic challenge modulating the proinflammatory response, triggering innate immunity and activating B and T lymphocytes (Zaidi MR et al, 2011)
- *IL-2* is a growth factor for T-cells and B-lymphocytes. In addition, it stimulates antibodies production and secretion; IL-2 participate with IL-12 to increase NK cytotoxicity (Garlanda C et al, 2013)
- *IL-4* is a cytokine that participates in the regulation of the immune system at multiple levels; in particular, IL-4 drives the expansion of B-lymphocytes clones and activates the alternative macrophage response (Luzina IG et al, 2012)
- *IL-6* is a multifunctional cytokine involved both in inflammation and infection responses inducing the synthesis of acute phase proteins. It is also involved in the regulation of metabolic and regenerative processes (Scheller J et al, 2011)

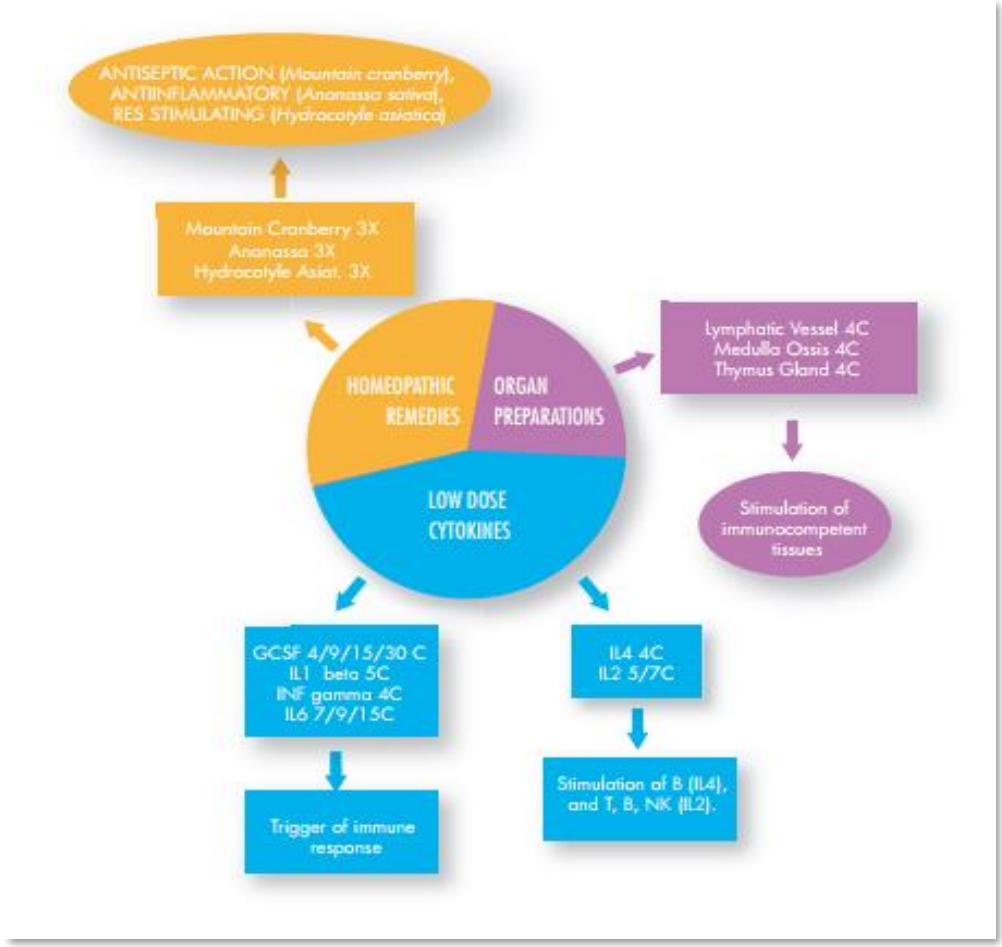

Figure 7: Citomix composition and description

The concentrations of the signaling molecules in citomix permit them to act within the complex network of signals supporting their overall response and maintaining system homeostasis. Low concentrations of signaling molecules show a safe pharmacological profile (Martin-Martin LS et al, 2017; Carello R et al, 2017); this aspect is fundamental for the clinical applications of citomix.

3) The components of animal origin are three swine organ derivatives (*Glandula Thymi suis, Medulla ossis suis, Vasa lymphatica suis*); They exert immunologic effects on the corresponding human organs, contributing to the modulation of inflammatory phenomena. The specific organotherapics concentrations ensure their correct processing by the APCs (Antigen Presenting Cells) expanding the regulatory lymphocyte clone (Heine H et al, 1998; Reinhart E et al, 2001).

2.2.6.2 *Evaluation of optimal drug concentration in culture*

Scalar drug concentrations were cultured for 5 days with PBMCs and AMCs in RPMI 1640 medium with10% fetal calf serum (FCS). The optimal drug concentrations were detected on the basis of better proliferative response after an incubation period of 18 hours with ^{3}H-thymidine and next determination of proliferation stimulation-index (SI) for each drug concentration of dose-response curve.

2.2.6.3 *Study of Natural Killer (NK) cells activity*

AMC of patients were isolated from adenoids after centrifugation on a Ficoll-Hypaque gradient. NK cytotoxic activity was evaluated after incubation of AMC with K562 cells (human chronic myelogenous leukaemia cells), previously labelled with ^{51}Cr (Perkin Elmer, U.S.A.).

AMC and K562 cells were incubated in RPMI 1640+10% FCS, for 4 hour. NK activity was determined as lysis percentage of K562 by NK cells, and was evaluated with a TOP Count (Packard).

2.2.6.4 *Evaluation of lymphocytes proliferative potential with CFSE*

AMC proliferative capacity in response to optimal citomix concentrations has been evaluated using CFSE (Carboxyfluorescein Succinimidyl ester) 1μM (Invitrogen) (Lyons et al, 2000).

Breefly, cells were incubated with CFSE 1μM, resuspended in RPMI 1640+ 10% FCS and plated into 96 well plates (Corning Costar, USA) with or without citomix, for 7 days at 37°C with 5% di CO_2.

After this incubation period, cells were marked with surface monoclonal antibodies, specific for the characterization of T CD4 e TCD8 lymphocytes. Percentage of proliferating T lymphocytes was evaluated using Navios Cytometer (Beckman Coulter, USA) and data were analyzed using Kaluza Software (Beckman Coulter, USA)

2.2.6.5 *3-day and 10-day ex vivo culture of AMC*

A suspension of AMCs ($1x10^{6}$ cell/mL) in culture medium was seeded in two 96-well tissue culture plates in presence of RPMI alone and RPMI plus citomix 1:10 (citomix). This working concentration was chosen after preliminary experiments (data not shown) using different concentration ratio of citomix in RPMI, in order to identify the optimal one to preserve cell viability and obtain adequate stimulation after 10 days.

After 3-days stimulation, cells were harvested, centrifuged and supernatants were collected for cytokine (IL-6, IL-10, IFN-γ) assay. After 10-days stimulation, cells were harvested,

centrifuged and supernatants were collected for cytokine and immunoglobulin assessment, and cells were labeled for the evaluation of B lymphocytes sub-populations.

2.2.6.6 *Quantification of Immunoglobulins and cytokine levels in 3 day- and 10 day-culture supernatants*

Microtiter plates were briefly coated with purified monoclonal antibody anti-human IL-6, IL-10 and IFN-γ (Endogen-Tema, USA). After stabilisation with 2% BSA (Bovine Serum Albumin, Sigma, USA) in PBS (Phosphate Buffered Saline, Euroclone, Italy) for 1h, the samples were added. Monoclonal anti-human biotinylated antibodies were used. Reproducibility and specificity of the assay were previously verified. The plate was read at 450 nm in a Sunrise microplate reader (Tecan, Switzerland) and the concentration of cytokines was extrapolated from a standard curve included in each plate and expressed in ng/ml. IgA, IgG and IgM levels in culture supernatants after 10 days in culture have been quantified as described above.

2.2.6.7 *Surface Phenotype Detection of B cell populations after stimulation with citomix*

AMC after 10 days culture with or without citomix were labeled with the appropriate monoclonal antibodies (mAb) which included: Fluorescein isothiocyanate (FITC), phycoerythrin (PE) PerCP o PC7-labeled anti-CD19, anti-CD27, anti-IgD (Beckman Coulter, USA). Appropriate isotype-matched controls were included. B cell populations were identified as follows: B naïve CD19+-IgD+-CD27-, B able to switch CD19+- IgD+-CD27+, and B switched CD19+-IgD--CD27+.

Cells were acquired by Navios flow cytometer (Beckman Coulter, USA) and analysis was performed with the use of Kaluza software (Beckman Coulter, USA).

2.3 Statistical analysis

For Part I, statistical analysis were performed using Stata 14.2 (StataCorp, College Station TX, USA). A 2-sided p-value < 0.05 was considered statistically significant; given the exploratory nature of the study no multiple test adjustments were used. Data were described as the mean and standard deviation (SD) or median and quartiles if continuous.

For Part II, categorical variables were described by frequency tables, while continuous ones were given as mean and standard deviation or median and range, based on their distribution. Differences within subject among continuous variables were analyzed with Student's t test for paired data if distribution, checked with the Shapiro-Wilk test, was found normal (also after nonlinear transformation, if needed), and if variances, checked with the Levene's test, were found

equal. If these conditions were not simultaneously obeyed, then the Wilcoxon test was used. Statistical significance was considered with $p < 0.05$.

2.4 Results

PART I

2.4.1 Patient characteristics

The 277 patients enrolled had a median age of 5.5 years (25th–75th 4.5–6.9 years). In the smoke exposed group, the median age was 6.0 years (25th–75th 5–7.3 years); in the non-exposed group the median age was 5.2 years (25th–75th 4.3–6.6 years). Among exposed children, the presence of one active smoker in the household was reported in 65% of cases, while in 35%, two active smokers were reported. In the atopic group, the median age was 6.0 years (25th–75th 5.1–7.7 years), while in the non-allergic group the median age was 5.2 years (25th–75th 4.3–6.3 years). We observed a positive association between age and passive smoke exposure, with a mean age ± S.D. of 5.76 ± 2.19 years for non-exposed children and 6.88 ± 3.11 for exposed ($p = 0.002$). A positive association was also observed between age and atopy, with 5.7 ± 2.31 for non-allergic and 6.77 ± 2.83 for allergic subjects ($p < 0.001$). Moreover, we found a positive association between age and the presence of both risk factors, 5.64 ± 2.26 with no risk factors, 6.01 ± 2.23 with only one risk factor, and 7.71 ± 3.40 with both risk factors ($p < 0.001$). No association was observed between gender and passive smoke exposure ($p = 0.796$) or atopy ($p = 0.208$).

2.4.2 T-independent Immunoglobulin production: changes between exposed and non-exposed patients

The *in vitro* evaluation of immunoglobulin production was performed on all enrolled patients, but in some cases data were not obtained due to a low cellular concentration. With the term "baseline" in Ig production after stimulation with TLRs-ligands, we intended Ig produced by AMC after 10 days in culture in RPMI 1640 medium + 10% FCS and IL2, without incubation with TLRs ligands. With the term "resting" in T-independent Ig production, we intended Ig produced by AMC after 10 days in cultures in Iscove's complete medium without incubation with mouse fibroblasts cell line CDw32 presenting mouse anti-human CD40.

Regarding smoke exposure, *in vitro* T-independent immunoglobulin production (Figure 8) was evaluated in 276 samples (84 exposed and 194 non-exposed). Basal levels were comparable between groups ($p = 0.658$, $p = 0.307$ for IgA and IgM respectively), except for IgG ($p = 0.001$); and within groups, changes from baseline were significantly higher. According to a regression

model adjusted for baseline values, stimulation induced a significantly higher immunoglobulin production in exposed patients compared to non-exposed ones, for all detected immunoglobulins.

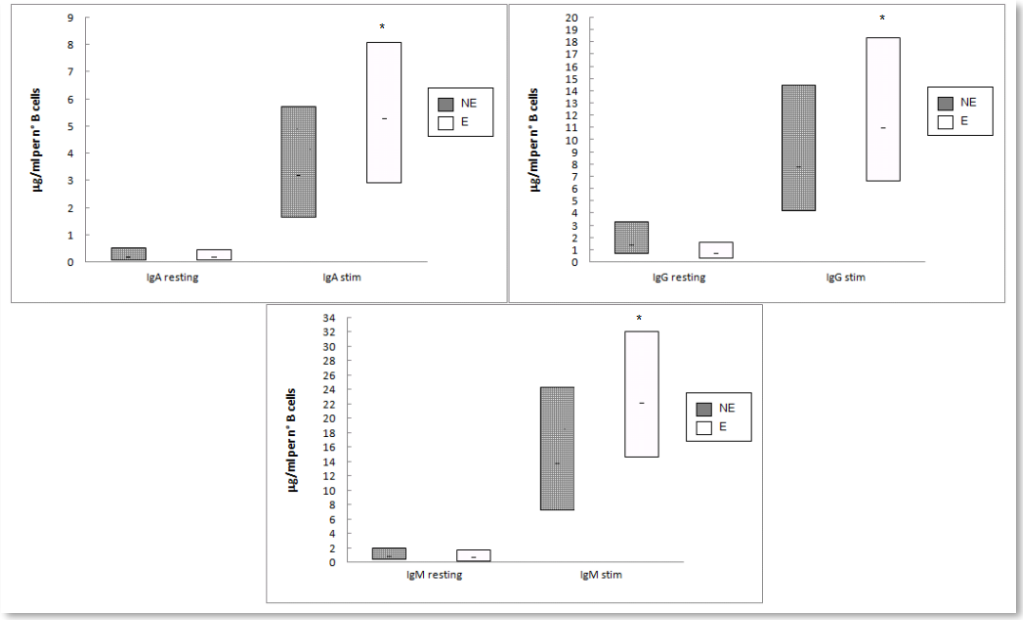

Figure 8: T-independent immunoglobulin production in exposed and not exposed group

Regarding atopy, T-independent immunoglobulin production was detected in 265 samples (97 atopic and 168 non-atopic). Basal levels were comparable between groups (p = 0.382, p = 0.659, p = 0.740 for IgA, IgG and IgM respectively); and within groups, changes from baseline were significantly higher. According to a regression model adjusted for baseline values, IgA levels were higher after stimulation in the atopic group, while IgG and IgM levels were higher in the non-atopic group, but significant differences were not observed between the two groups (Figure 9).

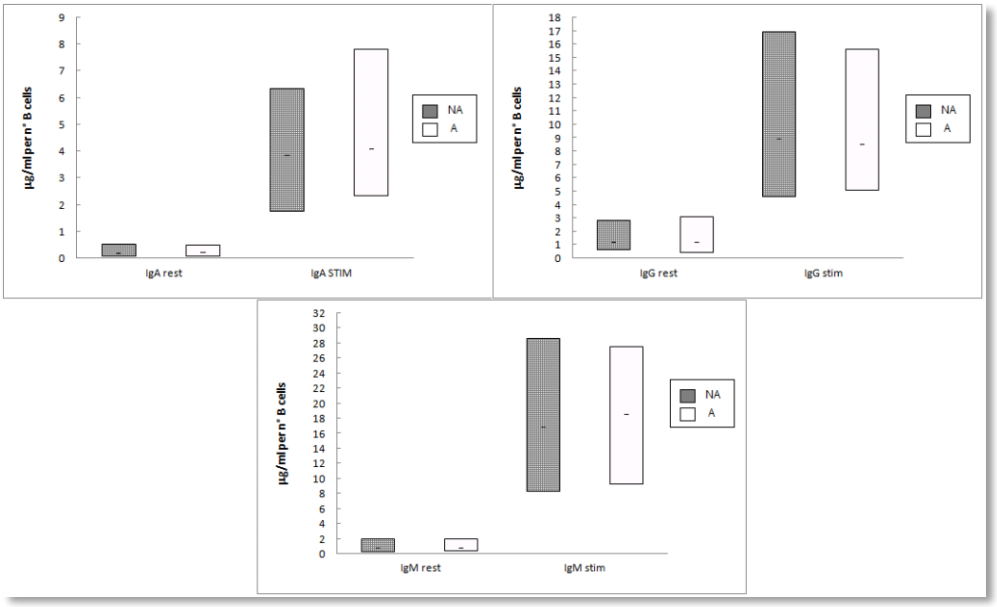

Figure 9: T-independent immunoglobulin production in atopic and non-atopic group

When combining both the presence of smoke exposure and atopy, in the T-independent immunoglobulin production we found a positive association between the number of risk factors and IgA and IgM production (Table 1).

Table 1 *In vitro* T-independent immunoglobulin production with respect to the number of risk factors (*differences in medians; Bold characters indicate statistically significant data (p≤0.05); 0= no risk factors, 1= one risk factor (smoke or atopy), 2= both risk factors)

	Risk Factor: Combined	IgA	IgG	IgM
Rest (median (25th-75th))	**0**	0.17 (0.07 - 0.54)	1.30 (0.68 - 3.08)	0.82 (0.38 - 1.99)
Stim(median (25th-75th))	**0**	3.19 (1.68 - 5.55)	7.90 (4.18 - 14.97)	14.71 (7.25 - 24.36)
***Δ* Rest-Stim** [95%CI]*	***0***	*-3.39 [-4.01 to -2.87] p<0.001*	*-6.97 [-8.38 to -5.77] p<0.001*	*-15.24 [-17.80 to -12.70] p<0.001*
Rest (median (25th-75th))	**1**	0.23 (0.08 -0.5)	1.40 (0.58 - 3.38)	1.00 (0.40 - 2.42)
Stim(median (25th-75th))	**1**	3.88 (1.80 - 7.40)	9.11 (5.32 - 17.70)	19.27 (9.30 - 30.90)
***Δ* Rest-Stim**[95%CI]*	***1***	*-4.01 [-4.84 to -3.19] p<0.001*	*-8.25 [-9.98 to -6.65] p<0.001*	*-18.10 [-21.93 to -14.61] p< 0.001*
Rest (median (25th-75th))	**2**	0.20 (0.10 - 0.45)	0.60 (0.30 - 1.40)	0.70 (0.24 - 1.24)
Stim(median (25th-75th))	**2**	5.10 (2.92 - 9.14)	9.30 (6.00 - 17.84)	21.11 (15.80 - 30.10)
***Δ* Rest-Stim**[95%CI]*	***2***	*-5.55 [-7.65 to -4.25] p<0.001*	*-8.62 [-10.87 to -6.67] p<0.001*	*-20.90 [-25.14 to -17.60] p<0.001*
Regression models adjusted for baseline		p= 0.436	p= 0.014	p= 0.629
ΔRest-Stim[95%CI], (log scale)	**1 vs 0**	0.07 [-0.22 to 0.36] p= 0.637	0.02 [-0.25 to 0.29] p= 0.877	0.06 [-0.25 to 0.37] p= 0.701
ΔRest-Stim[95%CI], (log scale)	**2 vs 1**	**0.45 [0.14 to 0.77] p= 0.005**	0.15 [-0.21 to 0.51] p= 0.416	**0.40 [0.09 to 0.72] p= 0.012**

2.4.3 T-dependent Immunoglobulin production: changes between exposed and non-exposed patients

The *in vitro* immunoglobulin production after TLR-ligands stimulation was performed on 273 samples (82 smoke-exposed and 191 non-exposed). Baselines were comparable (p = 0.488, p = 0.257, p = 0.348 for IgA, IgG and IgM respectively) and, within groups, changes from baseline were highly significant. Regarding smoke exposure, we observed the same trend in IgA, IgG and IgM production both in exposed and non-exposed patients, with a reduction after CpG stimulation and an increase after LPS stimulation. Even if no significant differences were observed between the two groups, immunoglobulin levels in non-exposed group were little higher, according to a regression model adjusted for baseline values (Figure 10).

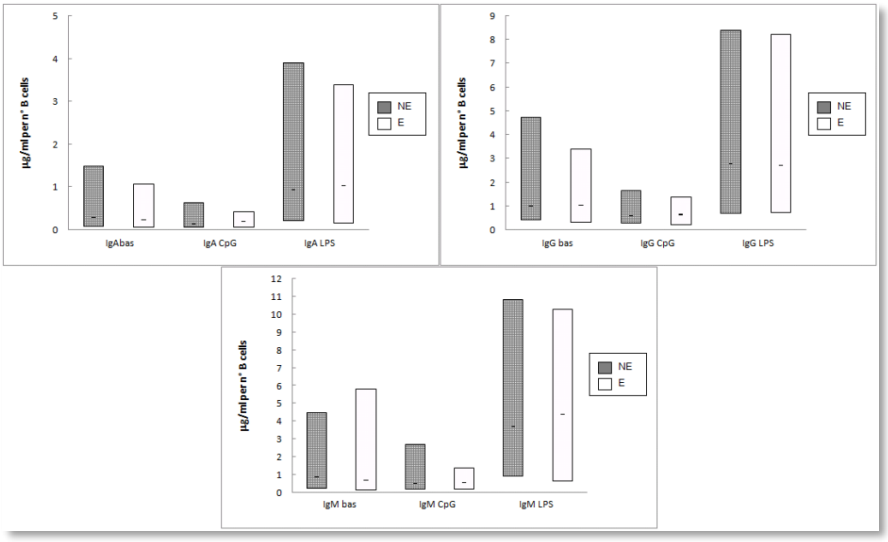

Figure 10:T-dependent immunoglobulin production in exposed and not-exposed group

In vitro stimulation with TLR-ligands was performed on 262 samples (97 atopic and 165 non-atopic). Baseline data were comparable ($p = 0.487$, $p = 0.256$, $p = 0.348$ for IgA, IgG and IgM respectively); and within groups, changes from baseline were highly significant. Atopic patients produced higher levels of IgA, IgG, and IgM compared to non-atopic patients, with the same trend in both groups after CpG and LPS stimulation. Ig levels decreased after CpG stimulation, while they increased after LPS stimulation in both groups. When comparing changes in immunoglobulin production after stimulation and according to a regression model adjusted for baseline values, we observed a significant difference between the atopic and non-atopic patients only for IgM, with a larger decrease in the atopic group after CpG stimulation (Figure 11).

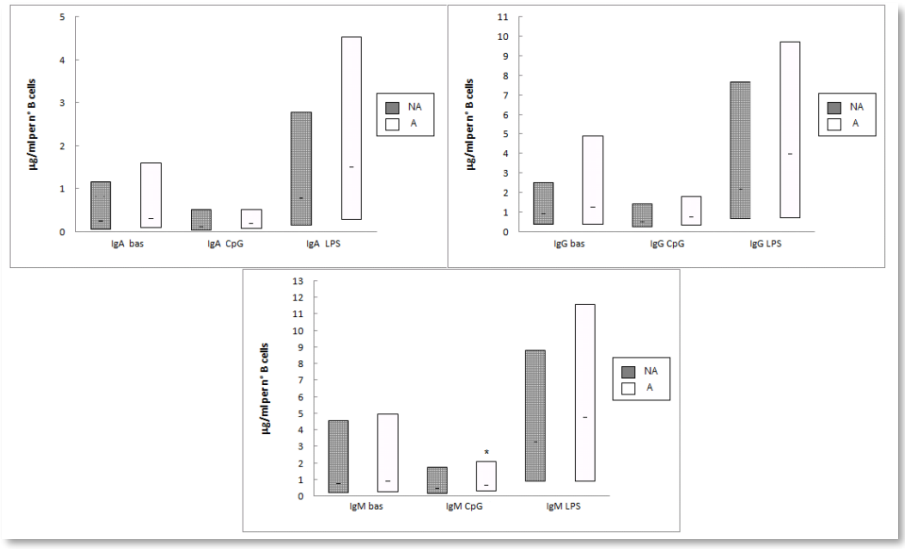

Figure 11: T-dependent immunoglobulin production in atopic and non-atopic group

When combining both the presence of smoke exposure and atopy, we observed that the CpG-induced decrease in IgA and IgM production was significantly associated with the number of risk factors (Table 2).

Table 2: *in vitro* immunoglobulin production after TLRs-ligands with respect to the number of risk factor (* differences in medians; Bold characters indicate statistically significant data (p≤0.05); 0= no risk factors, 1= one risk factor (smoke or atopy), 2= both risk factors)

	Risk Factor: Combined	IgA	IgG	IgM
Bas (median (25th-75th))	0	0.27 (0.07 -1.08)	0.93 (0.40 - 2.45)	0.76 (0.24 - 3.50)
CpG(median (25th-75th))	0	0.11 (0.05 - 0.48)	0.49 (0.27 - 1.13)	0.40 (0.17 - 1.23)
LPS (median (25th-75th))	0	0.83 (0.19 - 2.7)	2.19 (0.70 - 7.57)	3.10 (0.90 - 8.50)
Δ Bas-CpG [95%CI]*	*0*	*0.15 [0.07 to 0.34] p<0.001*	*0.52 [0.34 to 0.89] p<0.001*	*0.42 [0.14 to 0.80] p<0.001*
Δ Bas-LPS [95%CI]*	*0*	*-0.41 [-0.69 to -0.23] p<0.001*	*-0.96 [-1.49 to -0.54] p<0.001*	*-2.00 [-2.72 to -1.33] p<0.001*
Bas (median (25th-75th))	1	0.32 (0.06 - 1.85)	1.25 (0.46 - 5.90)	0.88 (0.28 - 5.70)
CpG(median (25th-75th))	1	0.26 (0.07 - 1.15)	0.80 (0.34 - 2.13)	0.90 (0.36 - 4.98)
LPS (median (25th-75th))	1	1.22 (0.22 - 4.42)	4.09 (0.80 - 9.68)	4.62 (0.96 - 12.06)
Δ Bas-CpG [95%CI]*	*1*	*0.09 [0.01 to 0.36] p= 0.033*	*0.54 [0.21 to 1.67] p<0.001*	*0.00 [-0.18 to 0.36] p= 0.965*
Δ Bas-LPS [95%CI]*	*1*	*-0.77 [-1.12 to -0.46] p<0.001*	*-2.30 [-3.23 to -1.49] p<0.001*	*-2.63 [-4.27 to -1.64] p<0.001*
Bas (median (25th-75th))	2	0.25 (0.09 - 0.9)	1.09 (0.19 - 3.87)	0.53 (0.10- 3.52)
CpG(median (25th-75th))	2	0.19 (0.12 - 0.34)	0.58 (0.23 - 1.40)	0.49 (0.20 - 0.90)
LPS (median (25th-75th))	2	1.12 (0.17 - 3.39)	2.36 (0.68 - 8.34)	4.50 (0.34 - 10.77)
Δ Bas-CpG [95%CI]*	*2*	*0.17 [0.04 to 0.48] p= 0.002*	*0.79 [0.28 to 1.34] p<0.001*	*0.50 [-0.05 to 1.33] p= 0.118*
Δ Bas-LPS [95%CI]*	*2*	*-1.06 [-2.10 to -0.45] p<0.001*	*-1.80 [-3.02 to -0.77] p<0.001*	*-4.15 [-6.00 to -1.96] p<0.001*
Regression models adjusted for baseline		p= 0.777	p = 0.858	p= 0.675
ΔBas-CpG [95%CI], (log scale)	**1 vs 0**	**0.48 [0.03 to 0.92] p= 0.037**	0.37 [-0.05 to 0.78] p= 0.086	**0.66 [0.14 to 1.17] p= 0.013**
ΔBas-CpG [95%CI], (log scale)	**2 vs 1**	**-0.50 [-1.00 to -0.0003] p= 0.05**	-0.33 [-0.90 to 0.24] p= 0.258	**-0.81 [-1.46 to -0.15] p= 0.017**
Δ Bas-LPS [95%CI], (log scale)	**1 vs 0**	0.41 [-0.07 to 0.89] p= 0.093	0.34 [-0.12 to 0.80] p= 0.142	0.23 [-0.26 to 0.73] p= 0.355
Δ Bas-LPS [95%CI], (log scale)	**2 vs 1**	-0.24 [-0.91 to 0.43] p= 0.48	-0.33 [-0.99 to 0.33] p= 0.324	-0.27 [-1.04 to 0.50] p= 0.493

2.4.4 *In vitro* detection of IL10-producing B regulatory (Breg) cells in adenoids

The presence of B10 regulatory cells has been detected based on their capacity to produce IL10 upon stimulation with CpG 2006 and CD40L, related to their atopic condition.

The CD19/IL10+ cells in adenoids were 8,50 [5–13.43] % of the total CD19+ cells. Notably, CD19/IL10+ cells frequency was higher in atopic than in non-atopic children, but this different is not statistically significant (p = 0.212) (Figure 12). In addition, we evaluated the expression of $CD1d^{hi}$, CD5, $CD24^{hi}$ on CD19/IL10+ cells. Atopic children had higher frequencies than non-atopic ones; in particular, there was significant difference (p = 0.003) for $CD24^{hi}$ CD19/IL10+ cells (Figure 12).

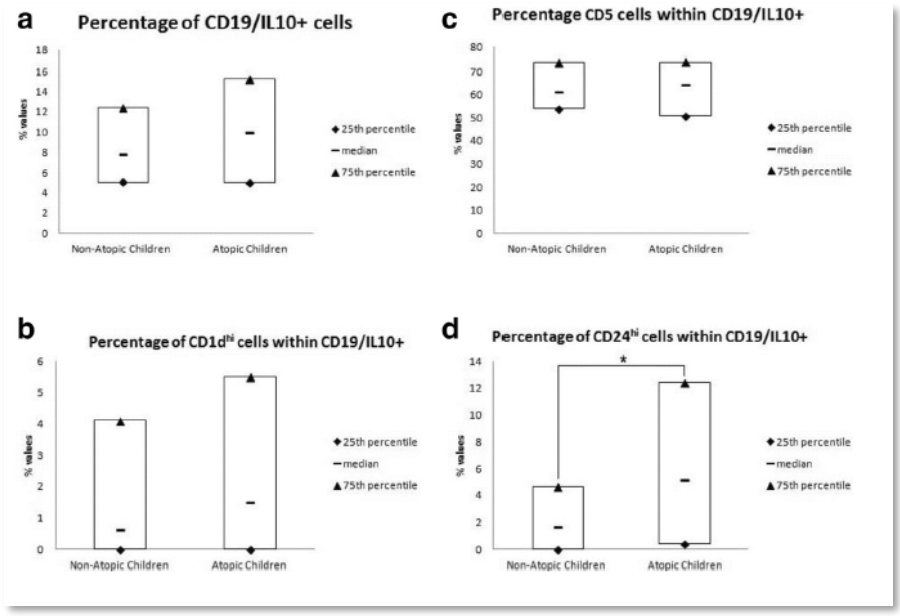

Figure 12: a Percentage of CD19/IL10+ cells expressed on the total of CD19+ cells; **b** percentage of CD1dhi cells expressed on the total of CD19/IL10 + cells; **c** percentage of CD5+ cells expressed on the total of CD19/IL10+ cells; **d** percentage of CD24hi cells analyzed within CD19/IL10+ cells expressed on the total of CD19/IL10+ cells. Comparisons between Atopic Children vs Non-Atopic Children: * p = 0.003 [Wilcoxon rank-sum (Mann-Whitney) test]

PART II

2.4.5 Evaluation of lymphocytes proliferative potential using CFSE

Evaluation of proliferative response of AMC after *in vitro* stimulation with Cytomix 1:10 and 1:100 leads us to declare that these two different concentration of the low medicine compoundare both safe and tolerated by the cells.

In particualr, at the adenoidal level, a low proliferative rate of T CD4 positive helper and T CD8 positive cytotoxic lymphocyte subpopulations is observed, consistent with the function of the adenoids of the germinating cell line of line B (Figure 13, Figure 14).

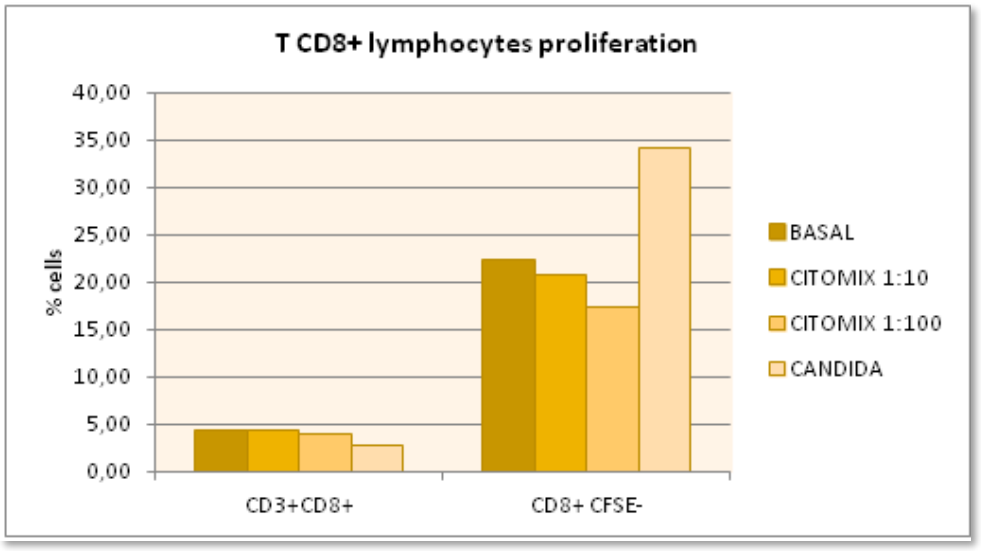

Figure 13: T CD8 positive lymphocyte proliferation with CFSE

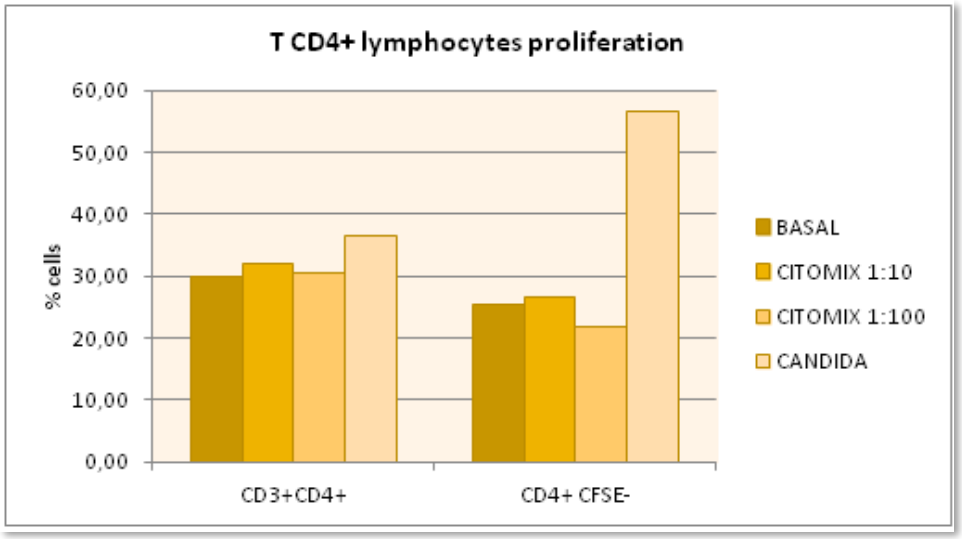

Figure 14: T CD4 positive lymphocyte proliferation with CFSE

2.4.6 Evaluation of Natural Killer (NK) cells cytotoxic activity

Adenoid cells could be considered as "superactivated" cells, due to their anatomical location and to their specific functions.

Although there is ad high intra- and intra-individual variability, we found a decrease in NK cells activity with citomix 1:10 in adenoid lymphocytes, with values that return to baseline levels with a immunomodulant product concentration of 1:100 (Figure 15).

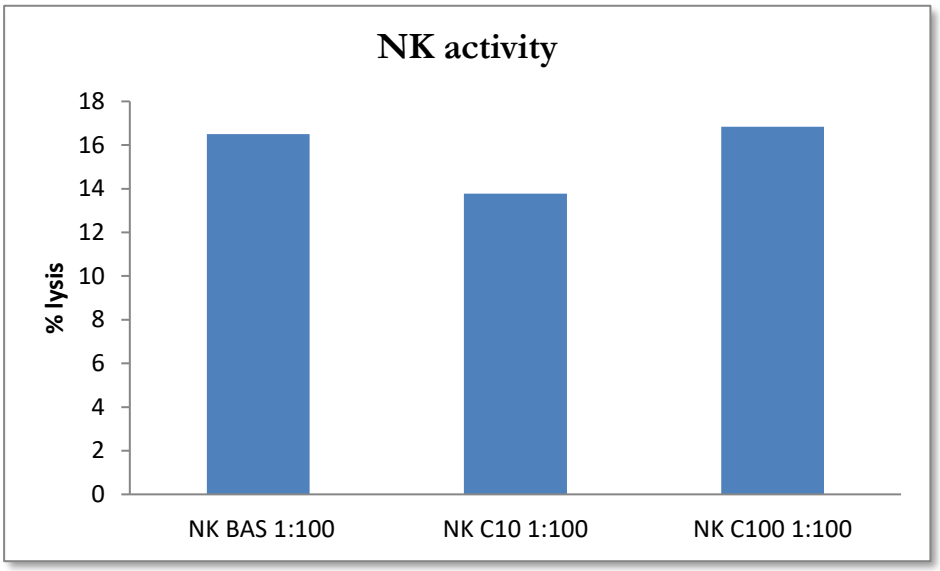

Figure 15: NK cells activity in adenoidal lymphocytes

We also performed a statiscal analysis in order to detect if there are significant differences between exposed and not-exposed patients. As sohwn in the figure below, no significant differences were detected between the two groups, neither for basal levels, nor after *in vitro* stimulation with both concentrations of citomix (Figure 16).

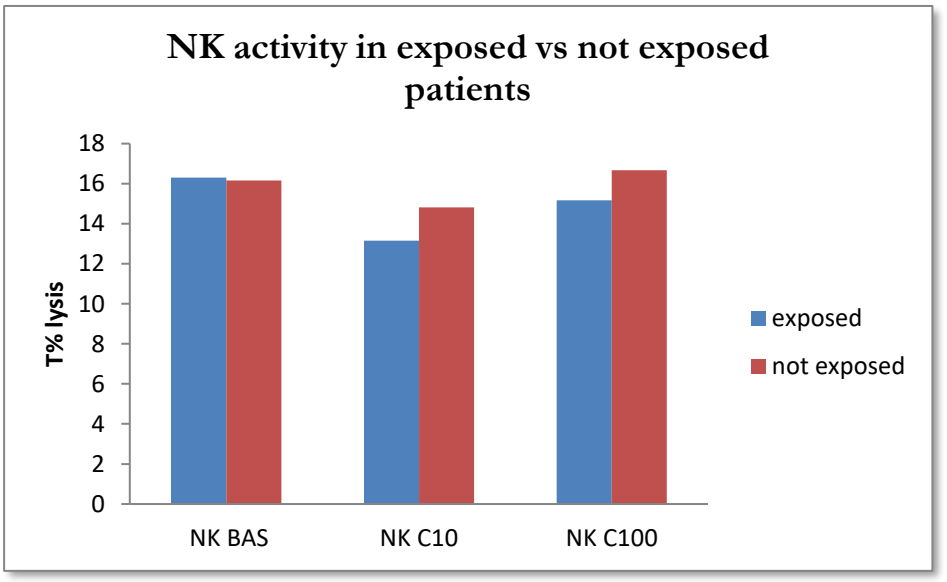

Figure 16: NK activity in exposed and not exposed group

Comparing the effects of citomix on NK activity between atopic and not atopic patients, we observed a decreased NK activity after stimlation with citomix 1:100 in not-atopic èatients, compared to atopic ones, but this difference is not statistically significant (Figure 17).

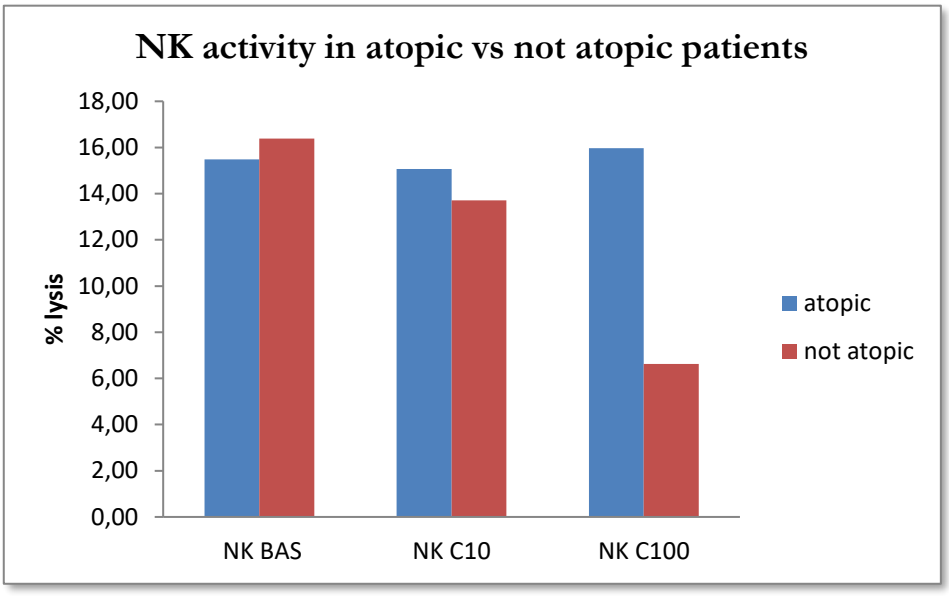

Figure 17: NK activity in atopic and not atopic group

2.4.7 Evaluation of cytokines and Immunoglobulins production

Citomix significantly increased IFN-γ concentrations in comparison with unstimulated conditions observed both after 3 days ($P<0.0001$) and 10 days ($P<0.01$) of culture (Figure 18).

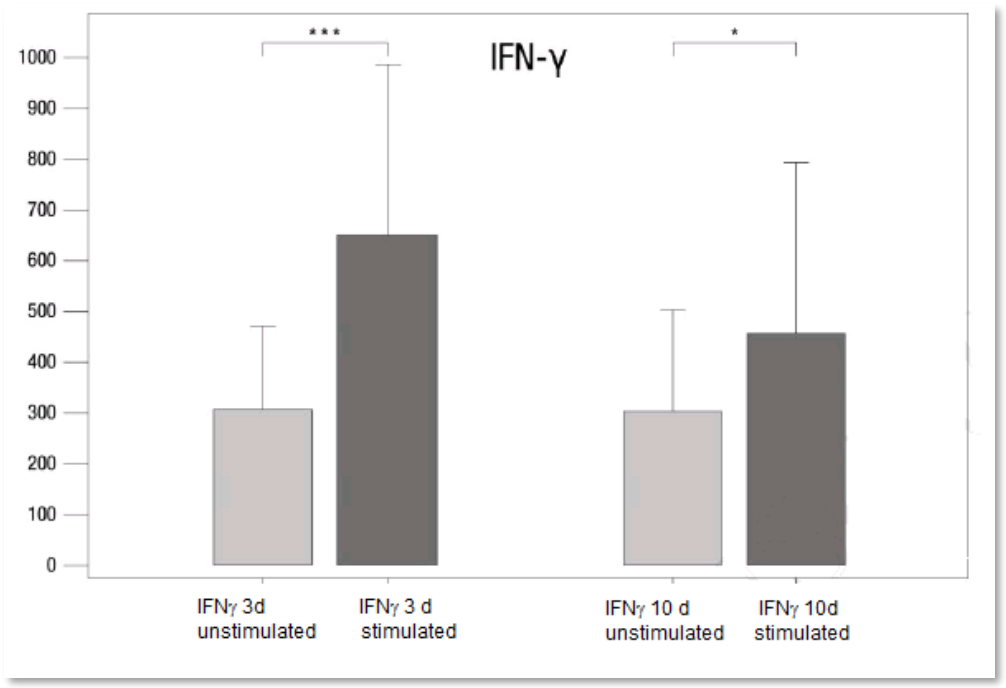

Figure 18: *ex vivo* IFN-γ production in 3-day and 10-day cultures, unstimulated or stimulated with 1:10 citomix. *=p<0.05; ***=p<0.0001

The comparison between IFN-γ levels in exposed and not exposed patients showed that in exposed patients the production of this inflammatory cytokines was lower than in not exposed group after stimulation with citomix. However, this difference is not statistically significant (Figure 19).

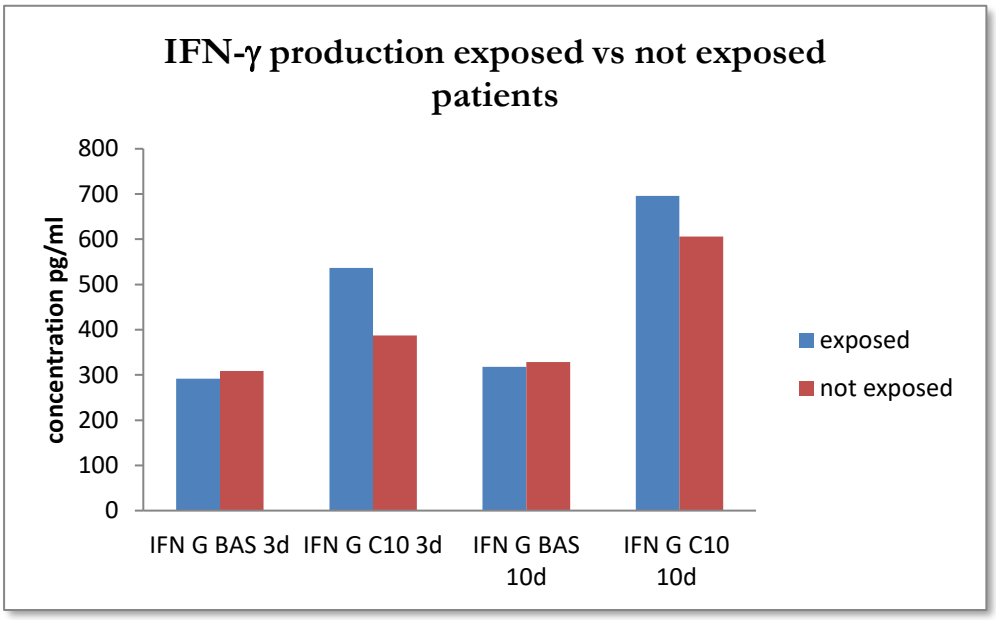

Figure 19: IFN-γ levels in exposed and not exposed group

Regarding the comparison between atopic and not- atopic group, we observed an increase in IFN-γ levels in not atopic group compared to atopics after 3 days of culture at basal level (p=0.042), while no differences between the two groups are observed after 10 days. No statistical signifcance was observed (Figure 20).

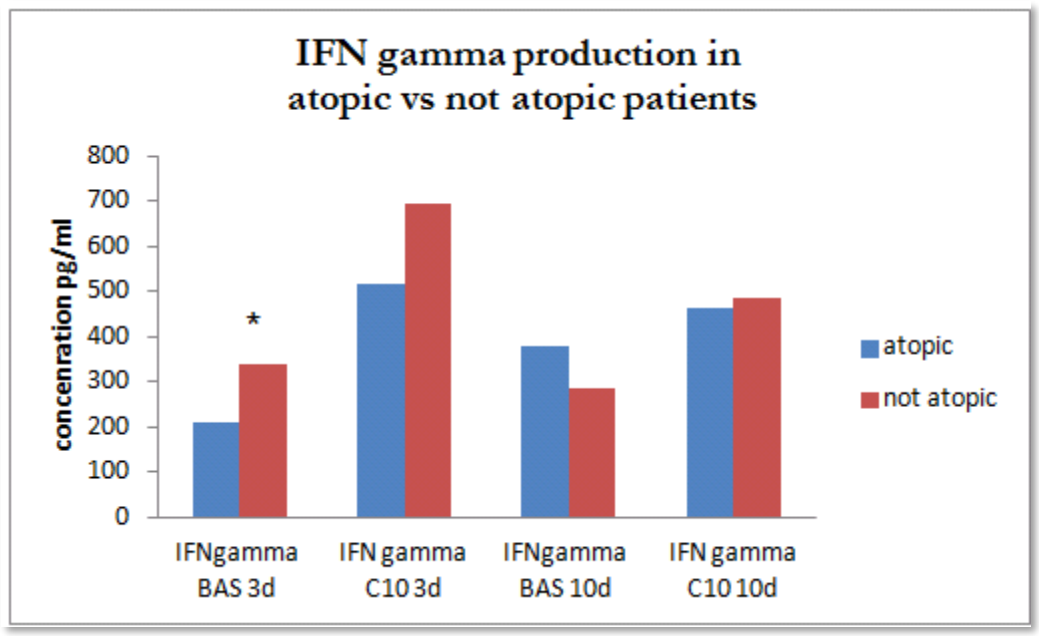

Figure 20: IFN-γ levels in atopic and not atopic group

Citomix significantly increased IL-6 concentrations in comparison with unstimulated conditions observed after 3 days (P<0.05), but not after 10 days (P=n.s.) of culture (Figure 21).

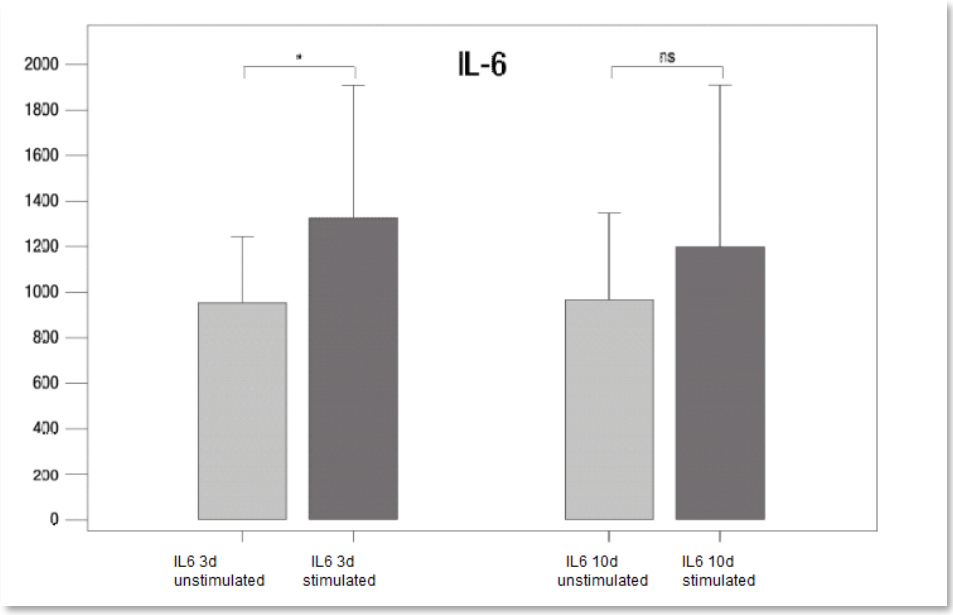

Figure 21: *ex vivo* IL-6 production in 3-day and 10-day cultures, unstimulated or stimulated with 1:10 citomix. *=p<0.05

Comparing the effects of citomix 1:10 between exposed and not-exposed patients, we observed that citomix leads to an increase in IL6 production after 3 days in cultures in both groups (compared to basal levels), with a higher increase in not exposed group. After 10 dayin in culture no differences were observed between the two groups (no statitical significance) (Figure 22).

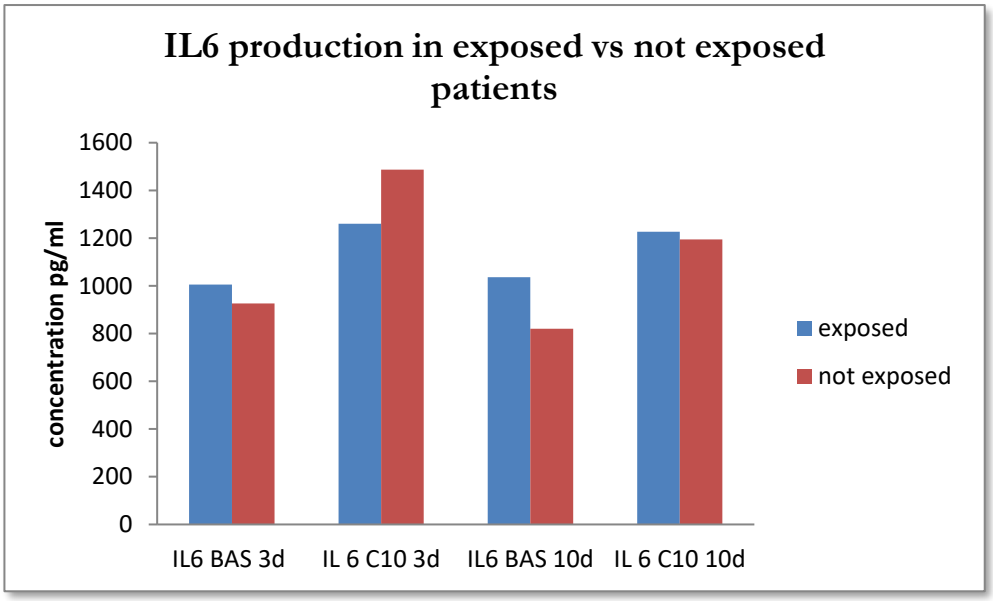

Figure 22: IL6 production in exposed and not exposed group

Regarding atopy, stimulation with citomix induced an increased compared to basal level only in the atopic group, while no differences were obserevd in the not atopic group at 3 days. After 10 days of culture, there is an increase of IL6 production only in the not atopic group. No statistical differences were observed between the two groups (Figure 23).

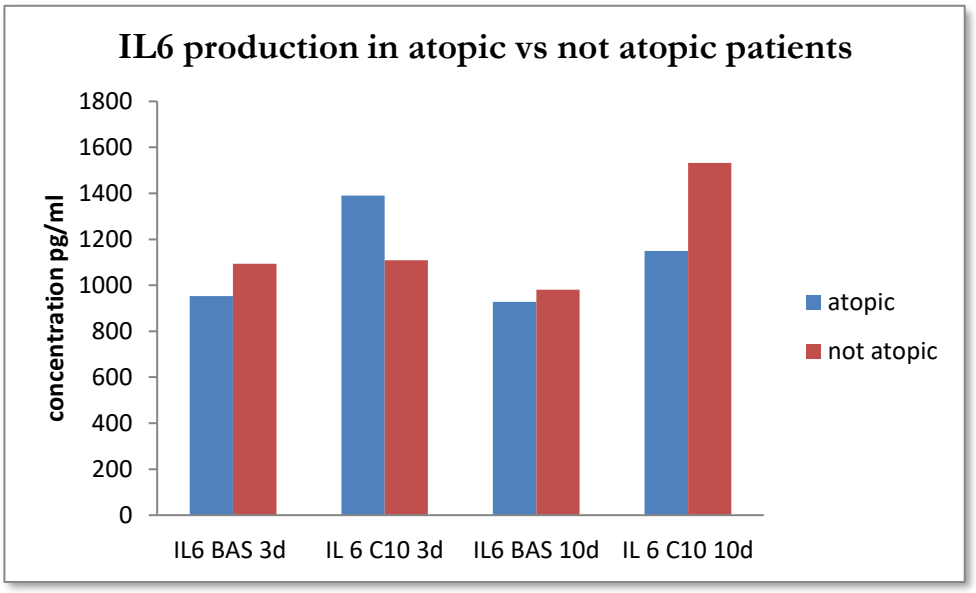

Figure 23: IL6 production in atopic and not atopic group

Citomix significantly decreased IL-10 concentrations in comparison with unstimulated conditions observed after 3 days ($P<0.05$) of culture (Figure 24).

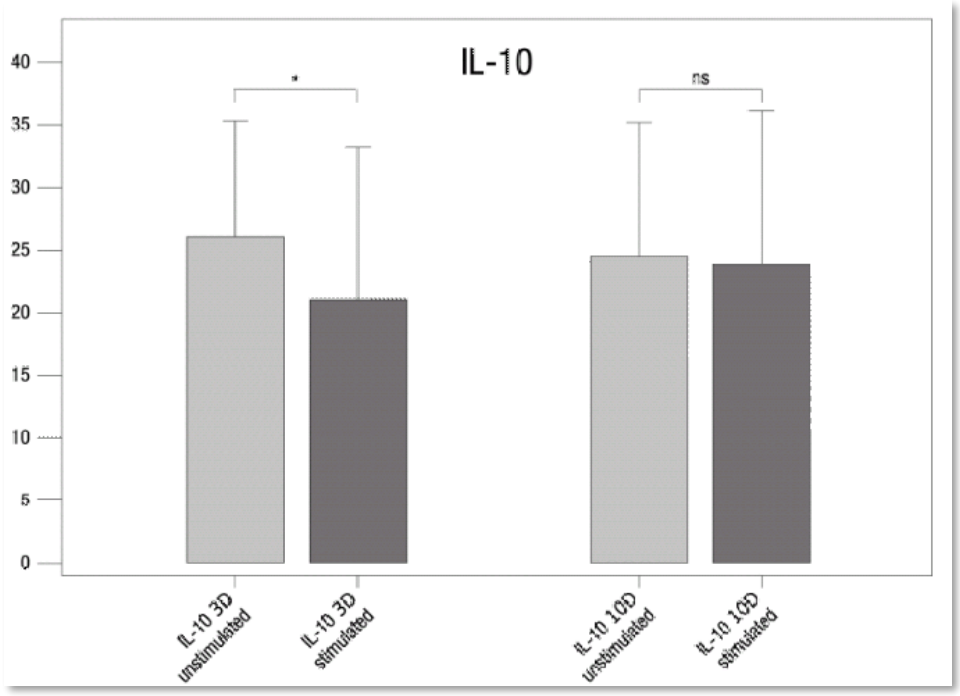

Figure 24: *ex vivo* IL-10 production in 3-day and 10-day cultures, unstimulated or stimulated with 1:10 citomix. *=$p<0.05$

The comparison between exposed and not exposed patients showed that after 3 days there is a decrease in IL10 production after stimulation with citomix, while no differences were appreciable in not exposed patients. After 10 days there is, on the contrary, an increase only in not exposed patients after stimulation with citomix. No statistical significance was observed between the two groups (Figure 25).

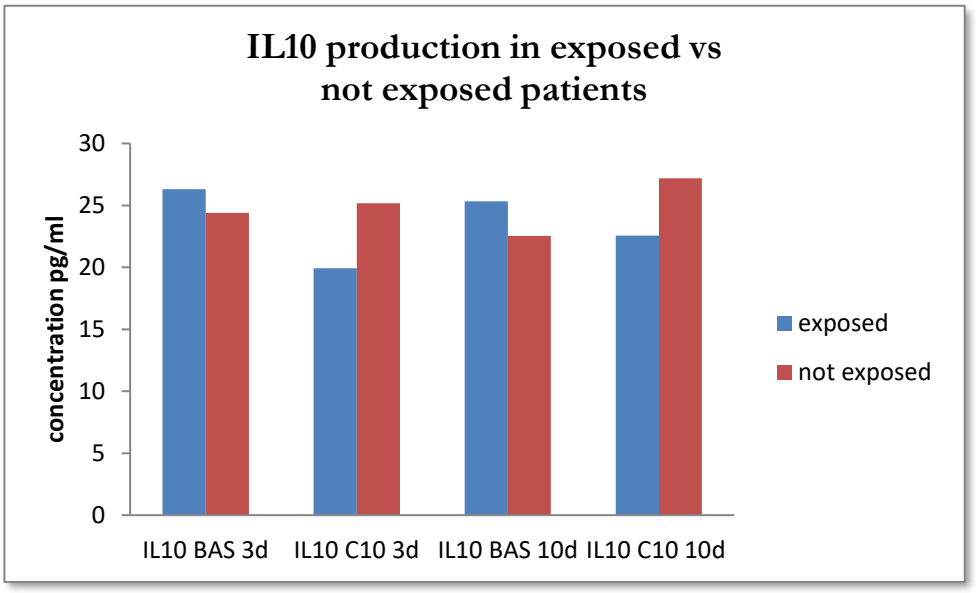

Figure 25: IL10 production in exposed and not exposed group

Regarding atopy, IL10 significanlty increased after 3 days in not atopic group (p=0.007) compared to atopics. After 10 days there is an increase in IL10 production in atopic groups, no appreciable differences were observed in not atopic patients. No statistical differences were detected between the two groups (Figure 26).

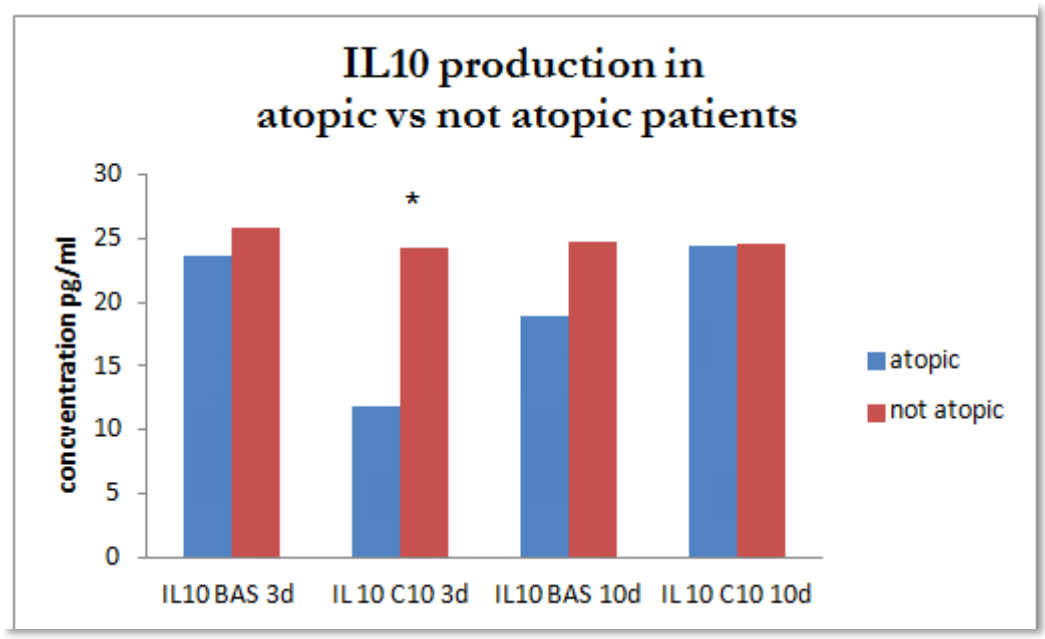

Figure 26: IL10 production in atopic and not atopic group

No particular differences were detectable for TNF-α concentration after stimulation.

Regarding Immunoglobulins production, citomix significantly increased IgA and IgM levels in culture suopernatants ($p<0.0001$ for both) compared with the unstimulated conditions at 10 days (Figure 27), while IgG production significantly decreased ($p<0.05$).

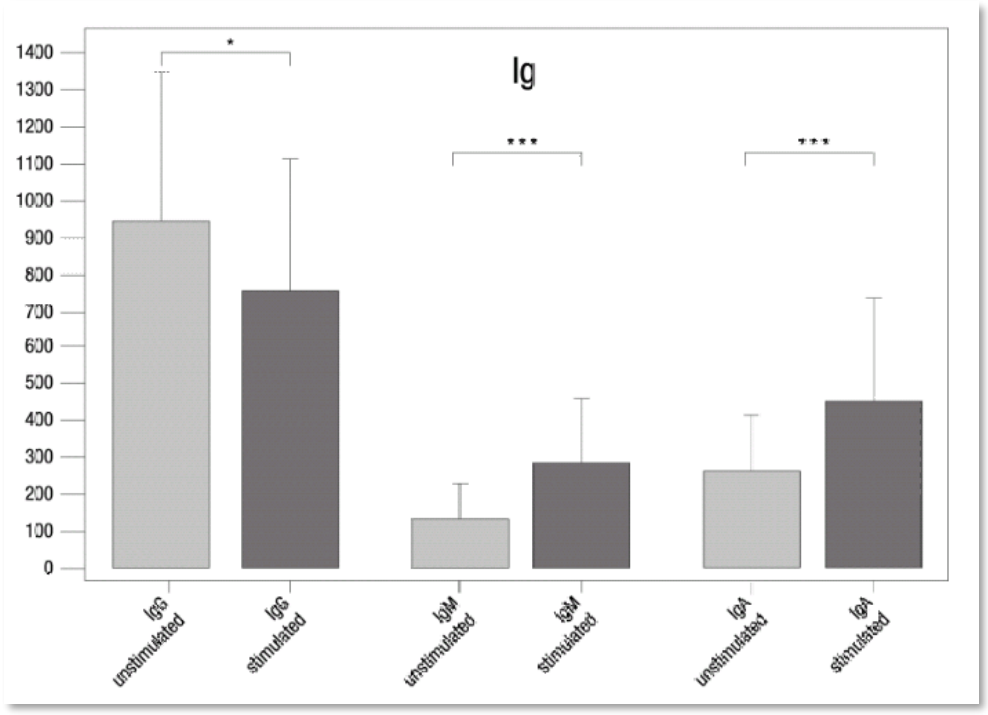

Figure 27: *ex vivo* Ig production after 10 days of culture, unstimulated or stimulated with 1:10 citomix *=p<0.05; ***=p<0.0001.

Making the comparison between exposed and not-exposed patients, IgA levels are lower in not exposed group than in exposed patients (with and without C10 stimulation) with a significant difference in the levels of IgA at basal levels between groups (p= 0.045). The same trand was observed in IgM levels, with a significant difference (p= 0.017) at basal levels between the two groups. No statistically significant difference were observed in IgG production between groups (Figure 28).

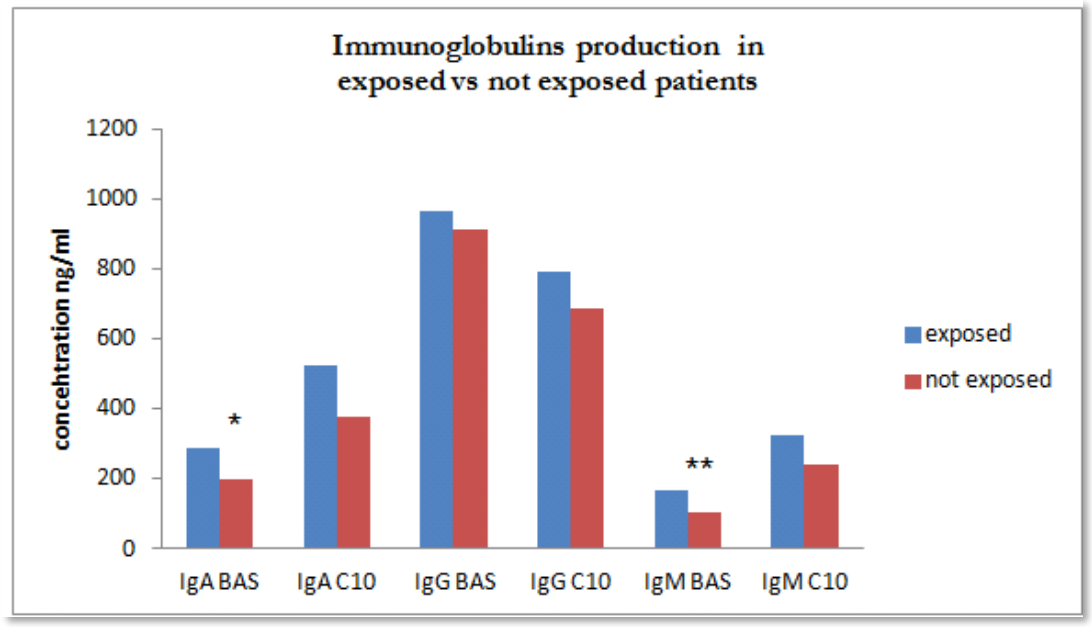

Figure 28: immunoglobulin production in exposed and not exposed group

Regarding atopy, the trend is a lower immunoglobulin production in not atpopic patients, compred to atopics, withot significant differences between the two groups. After stimulation with C10 ther is an increase for IgA and IgM levels and a decrease for IgG levels in both groups, without a statistically significant difference between the two groups (Figure 29).

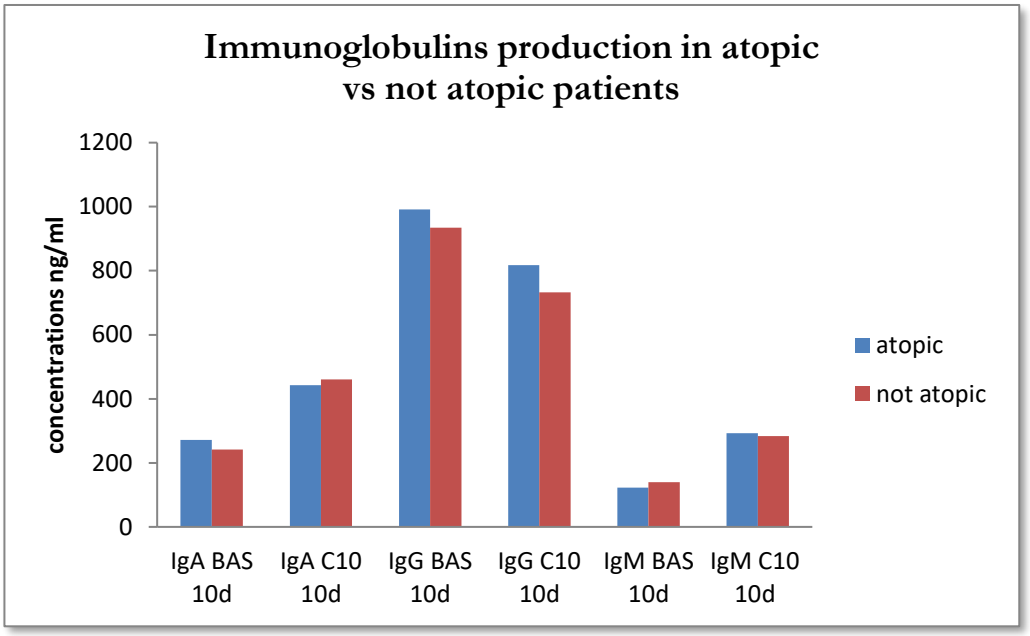

Figure 29: immunoglobulin production in atopic and non atopic group

2.4.8 Surface Phenotype Detection of B cell populations after stimulation with citomix

Analysing B cell populations and maturation, there was a significant increase in the B naïve (P=0.0005), B able to switch (p=0.0364) and B switched population (P=0.0003) in presence of citomix, compared with unstimulated conditions (Figure 30).

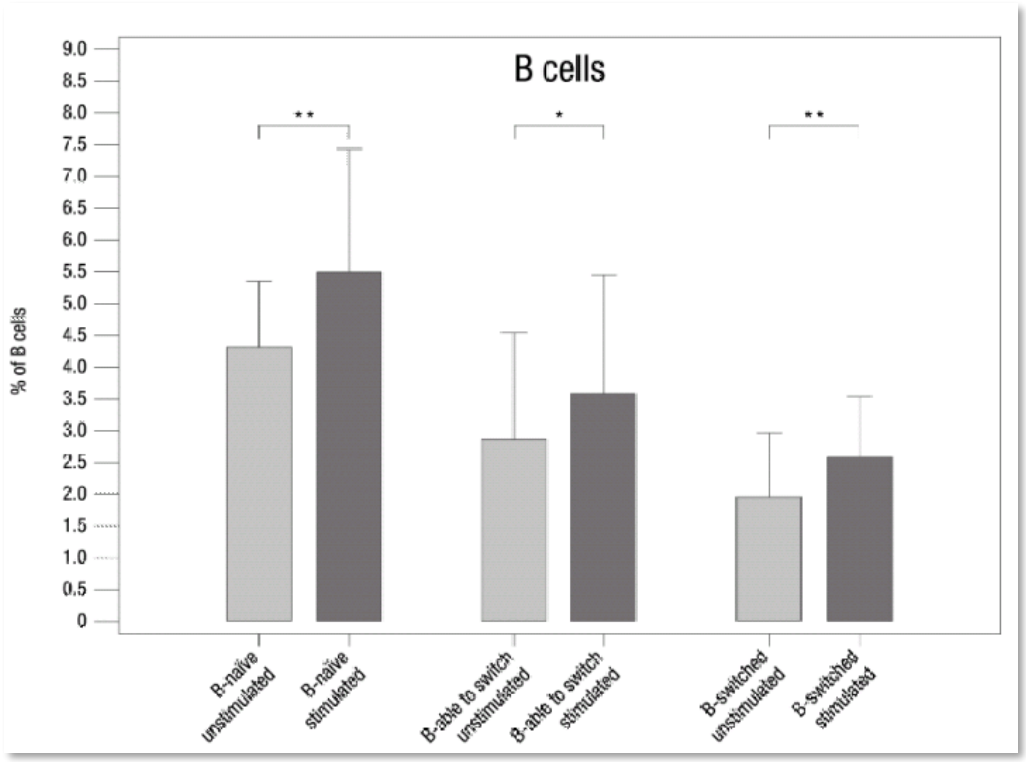

Figure 30: B cell sub-populations frequency of adenoidal cells, unstimulated or stimulated with 1:10 citomix. **=p<0.001

Making a comparison betweeen exposed and not exposed patients, there is a little increease in B naïve and B switched lymphocytes in not exposed groups compared to exposed after C10 stimulation, while there is a decrease in B able to switch population afte C10 stimulation in not exposed patients compared to the exposed. These differences are not statistically significant (Figure 31).

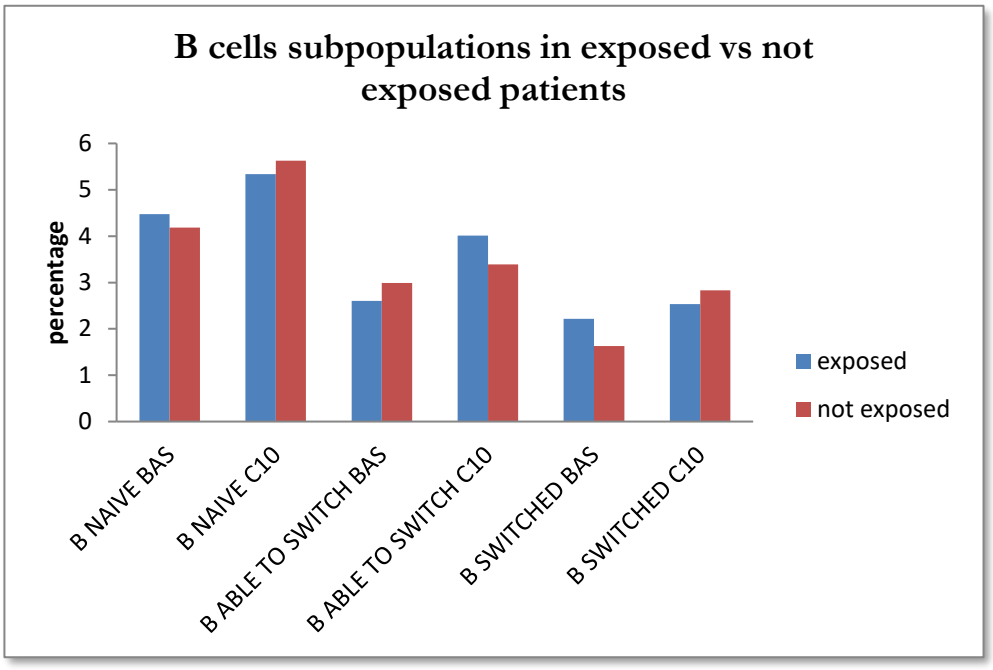

Figure 31: B cells subpopulations in exposed and not exposed group

The comparison between atopic and not atopic patients showed that there is an increase in all B lymphocyte subpopulatrions the in both groups after C10 stimulation. B naïve and B able to switch cells are higher in not atopic group but these differences were not statistically significant (Figure 32).

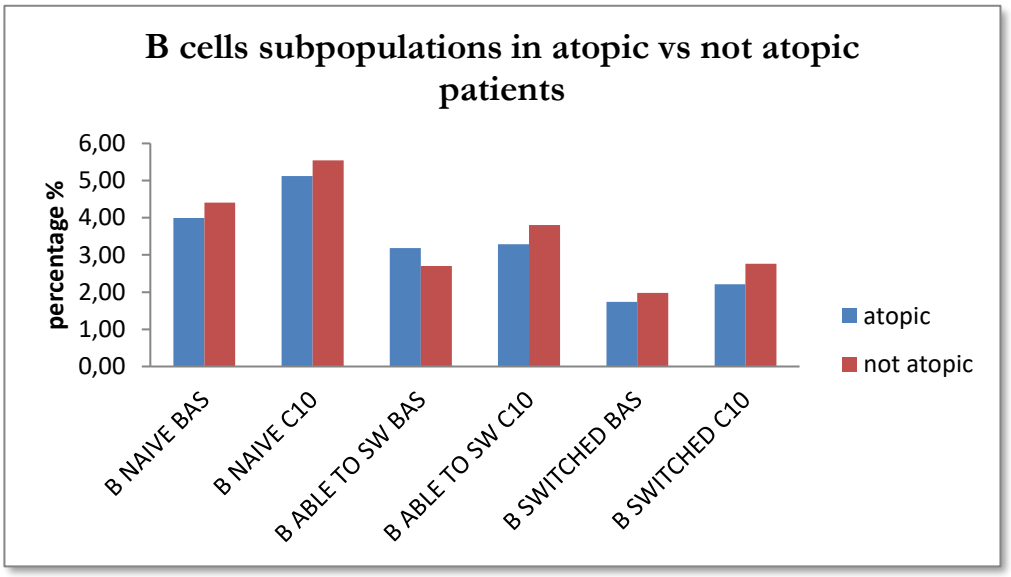

Figure 32: B cells subpopulations in atopic and not atopic group

3 DISCUSSION

Several different systematic analyses have been published about the immunodepressive effect of passive smoking. Passive smoke favours the onset of allergopaty and increases the risk of infections, especially in children. Atopy is in turn considered an important predisposing factor for the development of chronic inflammation and recurrent infectious diseases of the upper respiratory tract; atopic effects are particularly emphasised in smokers and in children exposed to passive smoke, also due to a dysbiosis of respiratory microbiota, induced by harmful constituents of cigarette smoke.

The anatomic district of the upper respiratory airways includes anatomically the adenoids, the two palatine tonsils and the lingual tonsils, that together constitute the lymphoid structure of Waldeyer's ring.

Owing to their peculiar localization, the adenoids are very exposed to both environmental (tobacco smoke and other air pollutants) and viral, mycotic and bacterial antigens, that lead to chronic state of inflammation, potentially responsible of adenoid hypertrophy, often requiring surgical removal111.

In this regard, we have focused the attention on the adenoidal immunity in paediatric patients undergoing adenoidectomy and we have in particular evaluated cellular and humoral response of adenoidal mononuclear cells in atopic/non atopic and exposed/non exposed to passive smoke children after *in vitro* stimulation with bacterial antigens. Moreover, in this work we have analysed the effect of *in vitro* stimulation with a low-dose medicine compound on adenoidal cellular response and we have finally made a correlation of these results with the eventual presence of atopy and passive smoke exposure.

In detail, in the first part of our research we have observed that in smoke exposed children there was an increased production of immunoglobulins (IgG, IgA, IgM) in an *in vitro* T-independent system., while no differences were observed after TLR stimulation (Tagliacarne et al, 2015). Even if this result coluld appear contradictory, we have speculated that chronic esposure to second-hand tobacco smoke might determine and maintain an inflammatory microenvironment leading to a persistent activation of antibody-producing cells. However, no appreciable direct effects of smoke were reported on adenoidal tissue or on local immune cells.

Regarding T-dependent immunoglobulin production, no appreciable differences were underlined between exposed and non exposed patients, but we have observed a reduced Ig production in all patients after CpG stimulation, probably due to TLR interaction in B-cells as explained by Hornung et al (Hornung et al, 2002).

The exact role of atopy and allergens in adenoidal hypertrophy is instead not fully clear, although is well known that adenoidal tissue of allergic children is more prone to recurrent respiratory inflammatory processes. In our data, no difference has been observed between atopic and non-atopic children in the T-independent *in vitro* model, while adenoidal lymphocytes of atopic children seem to better respond to TLR stimulation, in comparison with non-atopic subjects. We have thought that this increased Immunoglobulin production could be explained with a greater and continuous stimulation of B cellular responses in a more inflamed context.

Furthermore, in order to confirm that the simultaneous presence of both passive smoke exposure and atopy could enhance their singular effects on the immune system and on the inflammatory status, we have evaluated in the same subjects the presence of both risk factors and we observed an increasing trend for IgA and IgM T-independent production associated with the presence of both risk factors. This reinforces the hypothesis that the adenoidal inflammatory microenvironment of both exposed and atopic children leads to an exaggerated B cell local response.

In order to better characterize the adenoidal cellular composition, we have investigated the eventual presence of a new lymphocyte subpopulation, called B regulatory cells, known for their capacity to produce IL10 and particularly important for the maintenance of immunological homeostasis and tolerance to antigens, including self-antigens. Breg cells represent an heterogeneous group of immunosuppressive cells subset with distinct phenotypic and functional properties, that could play a role also in the induction of immune tolerance to allergens. With this study we indentify for the first time the presence of B-10 regulatory cells in adenoids of children with adenoidal hypertrophy and recurrent respiratory infections.

In particular, the percentage of B-10 cells was higher in atopic patients, probably because of the fact that their regulatory activity on other immunocompetent cells in lymphoid organs may be different than their regulatory activity in peripheral blood. This could be associated with IL10's ability to inhibit chemokine and proinflammatory cytokine production and IL10's ability to down regulate co-stimulatory molecule expression in APCs, in accordance with Komlosi et al., 2017 (Komlosi et al, 2017).

We also observed greater percentages of all investigated surface markers in allergic patients, significantly for $CD24^{hi}$ CD19/IL10+ cells. CD24 controls T and B cell maturation and function

and it could both improve and inhibit immune tolerance, regulating the efficiency of clonal deletion (Israel et al, 2005)

We have postulated that the percentage of $CD24^{high}$ cells is related to the IL-10-producing B cells in the$CD24^{high}$ $CD38^{bright}$ subpopulation percentage, but no detectable percentages in $CD1d^{hi}$/CD5+ and $CD24^{hi}$/$CD38^{bright}$ double positive cells were observed in both groups (data not shown). CD24 expression is usually associated with higher incidence of autoimmunity, even thought it has not been demonstrated its involvement in allergy development. However the higher CD24 percentage in atopic patients could be explained by the fact of in our study hypertrophic adenoids have been removed as consequence of recurrent and severe local infections.

In the second part, we have tested the activity of a multicomponent medication (citomix) on adenoidal immune response.

Our results have demonstrated that citomix was able to consistently increase IgA and IgM production and expand B memory cells. Moreover, citomix increases IL-6 and IFN-γ *in vitro* production and reduces early IL-10 release by adenoidal cells.

In particular, IL-6 is a cytokine, produced by macrophages and T cells to promote and amplify the immune response to infections, with multiple functions such as pro-inflammatory and anti-inflammatory actions; it is involved in both innate and adaptive immunity, stimulating the production of acute phase proteins as well as of antibodies (Wolf J et al, 2014). About the latter issue, IL-6 significantly affects Ig synthesis: the documented IL-6 increase could therefore explain the findings concerning the IgA and IgM raised production by citomix.

IFN-γ, released by activated B and T cells, takes usually action against viral infections by multiple effects, including strengthening of antigen presentation, phagocytosis, promotion of immunoglobulin production, and T helper switching (Kak G et al, 2018). Therefore, it seems to be substantially relevant the positive effect sustained by citomix during the events' cascade in immune response to infections.

IL-10, produced by several cells of innate and adaptive response, including macrophages, dendritic cells, natural killer, T and B cells, is pleiotropic in controlling viral immunity mainly concerning the prevention of damage deriving from exaggerated adaptive response (Ouyang W et al, 2011). However, virus may be able to exploit the IL10 functions by some mechanisms, such as immune evasion, suppression, and tolerance, so virus may contribute to its own survival and to chronic infection. IL-10 should be considered a "gianic" cytokine that may exert positive, but also negative effects that depend on time, pathogen load, via of entering of pathogen, host defence. In addition, IL-10 has a crucial role in switching the IgM and IgG classes. Therefore, the current

outcome could be useful to understand the potential mechanism of action of citomix during infection.

The comparison between citomix effect in atopic/not atopic and exposed/non exposed patients has shown a different cellular response to this low-dose medicine compound. in the groups. In detail, about atopy, is interesting to note the increasing trend of IL6 and IFN γ production in not atopic group after *in vitro* stimulation with citomix; moreover, immunoglobulin production and B cells subpopulations are globally higher in atopic group, but the effect after citomix stimulation is the same in both groups. About passive exposure to tobacco smoke, IFNγ production is in general higher in both group, but we have noted a more consistent increasing in exposed patients after *in vitro* stimulation with citomix and this result is probably dependent to more frequent viral infectious diseases and more pronounced inflammatory pattern in exposed children rather than not exposed ones; it is interesting to note that IL6 production is better stimulated by citomix in not exposed patient but only after trhee days of incubations, probably for a stronger effect of this compound on innate immunity cells; immunoglobulin production is globally higher in atopic group and after citomix stimulation we have observed a decreasing trend in both group for IgG only; is useful to underline that citomix stimulation is able to increase the percentage of B naïve and B switched particularly in not exposed patients.

Moreover, no significant differences have been observed after *in vitro* stimulation with citomix on NK cytotossic activity. This could be probably due to the absence of citomix direct effect on these cells.

Globally, these findings have given evidence that citomix could predictably promote a more efficient response to infections.

At this point, on the basis of our results, we can recognize the unquestionable immunomodulant activity of the used low-dose medicine compound. However, we have to stress the reciprocal influence of the local and systemical inflammatory status of the children enrolled: that's why the effect of citomix in exposed/not exposed and atopic/not atopic patients are a bit different from what was expected . Similarly, our analysis has been performed on local immune tissue, surgically removed because of chronic inflammation: this fact probably explains why we have found an increased production of inflammatory cytokines in not atopic and not exposed children also at the baseline, without citomix stimulation.

4 CONCLUSIONS

In conclusion, this is the first study that investigates *in vitro* adenoidal B cell response after different stimuli and that demonstrates the presence of B10 regulatory cells in adenoids of children with adenoid hypertrophy determined by chronic inflammation.

Considering their condition of exposure to passive smoke and atopy, these results, together with the comprehension of B regulatory mechanisms of action, could be useful in order to define new possible cellular markers for allergic inflammation monitoring and new therapeutic targets for improve treatment strategies.

Also on the basis of citomix activity, the goal could be the identification of new therapeutic tools and suitable subgroups of patients affected by adenoid hypertrophy which could retard or avoid surgical adenoidectomy and its correlated risks, thanks for example to citomix administration.

Performing analysis on local inflamed tissue only could surely represent a limitation of the study, owing to the lack of immunological status systemic overview and predictable influences of this local inflammatory state on *in vitro* cellular response. Unfortunately for this work we couldn't conduct analysis on peripheral blood cells because of ethical limitations.

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