“LUCIAN BLAGA” UNIVERSITY OF SIBIU

Faculty of Medicine

Doctoral Thesis

Assessment of allergic inflammation markers and immunomodulation in bronchial asthma

Abstract

Scientific coordinator,

Prof. Univ. Dr. MIHAI LEONIDA NEAMȚU

PhD Student,

Adriana Popescu

SIBIU

2015

Introduction

In this moment, over 20% of the global population suffers from some form of allergy with a prevalence that tends to increase. Although the exact etiology remains ambiguous regarding the allergic diseases, most researchers believe that the essential factor in the onset of allergies is represented by an epiphenomenon of exposure to the environment.

In this study, we started from the premise of hygiene hypothesis, according to which, the increase in the prevalence increasing allergic diseases occurs due to a decrease in exposure to microbes in early in life. The contact with microorganisms in the perinatal period is correlated with epigenetic regulation of genes involved in allergic inflammation, leading to decrease of probability thus developing certain allergic diseases.

By early contact with microbes in immune and metabolic programming, even during the fetal and childhood period, appear new opportunities to improve the health of pediatric patient and to reduce subsequently the risk of exposure to various diseases. According to the hygiene hypothesis, early development of the immune system is influenced both qualitatively and quantitatively by the microbiota bacteria. Intestinal microbiota is extremely variable in response to diet and environmental factors and, at the same time, governs a number of aspects of immune function in the body. The role of bacteria in the intestinal flora in modulating the balance of Th1/Th2 is demonstrated herein, as a result of striking a balance between pro-inflammatory cytokines and anti-inflammatory.

A great part of the researches on allergic diseases have focused on intestinal microbiota, allergic diseases in children being partially associated with imbalances in the intestinal microflora. In the case of dysbiosis, rebalancing the microbial balance can be achieved by administering probiotics,, live microorganisms administered in adequate amounts confer a health benefit for the host".

Asthma is based on adjusting the balance of Th1/Th2 in the direction of augmented production of Th2 cytokines. The interest in the therapeutic potential of probiotics in the case of allergic diseases is revealed from the fact that they enhance the intestinal permeability and reduce the level of inflammatory cytokines. According to the latest research, altered production of Th2 cytokines, by the induction of Th1 responses can occur both in prophylaxis and also in the treatment of allergic diseases.

The use of probiotics in preventing or treating allergic pathology is a new concept. Studies in recent years have been focused on a potential pediatric nutrition supplements with probiotics, some studies demonstrating control of inflammatory responses, a change in the cytokine profile, in the spirometric parameters, or clinical evolution in allergic patients of pediatric age[1].

Objectives

The main objective of this paper was the study of serum markers of allergic inflammation in patients with occasional wheezing, recurrent and especially asthma and also the observations of induced alterations on these markers of immunomodulatory treatment with probiotics. In this respect, in the personal study I intended to evaluate all relevant factors that develop asthma and the markers of allergic inflammation in patients with asthma and dynamics of Interleukin-4 (IL-4), interferon gamma (IFNγ) Serum eosinophil serum and Total IgE in these patients after treatment with probiotics. Subsequently to this central objective, we aimed to achieve the statistical processing of important parameters in conditioning the diagnosis of asthma and the dynamics change of these parameters under treatment with probiotics. The present study has a prospective character in terms of patient selection, investigation and prosecution of their therapy.

Methods

To achieve the proposed objectives, was selected a group of 136 children of both sexes diagnosed with occasional wheezing, recurrent and asthma in Sibiu Pediatric Hospital, Clinic of Pediatrics, 2013-2015. Patient recruitment was conducted under the study protocol approved by the Ethics Committee for Clinical Trials, of Clinical Hospital of Pediatrics.

In these patients there was made a clinical and para-clinical evaluation to establish the diagnosis of wheezing or asthma as recommended in the PractallGuides (2).

The 136 patients, selected according to randomization and who met the inclusion criteria, were formed in a batch in which were evaluated the relevant factors to the development of asthma and the markers of allergic inflammation in patients with asthma (Study I);

Of the 136 patients, enrolled in Study I, 80 were admitted to the study II, the rest did not meet the compliance criteria needed to complete the study due to withdrawal from the study due, on their own initiative.

Patients included in the II study, were given daily for four weeks a combination of probiotics. Children under 2 years received one capsule per day, those between 2 and 12 one capsule two times a day, following the administration of preparation, according to age.

Biological evaluation of patients who received immunomodulator treatment with probiotics included the determination of blood eosinophilia, by determining the peripheral blood picture and the total immunoglobulin E. In order to evaluate the allergic inflammation was determined in serum the plasma levels of interleukin-4 (IL-4) and Interferon gamma (IFNγ). Laboratory measurements were made at the inclusion in the study and were repeated after four weeks of treatment. I chose to evaluate these parameters before and after administration of probiotics, because they can define the allergic and inflammatory profile, their correlation can be really useful in shaping asthma diagnosis in an appropriate clinical context.

The patients received a combination containing 3 probiotic strains of bacteria. Each capsule contains 5.04 mg of freeze-dried bacteria (at least 1.2×10^7 units forming colonies in which: 4.5×10^6 Lactobacillus acidophilus 3.0×10^6, Bifidobacterium infantis, 4.5×10^6 Enterococcus faecium). Excipients, capsule content: lactose, dextrin, potato starch, magnesium stearate, the capsule shell: hypromellose, titanium dioxide, gelling agents and water.

Patients met the inclusion and exclusion criteria to reduce the influences of other phenomena on markers of allergic inflammation.

Criteria for inclusion in the study: under 14 years, informed consent to be admitted in the study, asthma diagnosed in history,wheezing history (occasional or recurrent);

Exclusion criteria of the study**:** parent’s or tutor’s refusal; congenital affections that evaluate with expiratory dyspnea (mucoviscidose, α1 antitrypsin deficiency, secretory IgA deficiency, diencephalic epilepsy, tracheobronchial dyskinesia, gastroesophageal reflux), malabsorption, immunological or oncological diseases, birth defects

Clinical examination. Focused on anamnesis, identifying allergising risk factors and physical examination in order to determine the severity of the disease. Based on detailed

anamnesis were obtained date from previous medical history of allergic nature, the presence of a family history of respiratory allergy, presence of other manifestations of atopy (respiratory, cutaneous), onset of symptoms.

Since asthma is a chronic inflammatory disease that involves many cells and mediators was considered useful the evaluation of certain parameters specific allergic evaluation allergic inflammation. Laboratory tests included the following parameters: automatic CBC with minimum 22 parameters, C-reactive protein, serum complement, electrophoresis of serum protein, immunoglobulins IgM, IgG, IgA, Total IgE, IgE-specific panel of 36 allergens Interleukin-4 and Interferon gamma-Fe, Ca total Mg.

The serum of the patients was analyzed by ELISA method (enzyme linked immunosorbent assay), for IgE total, IL-4 and IFNγ. To determine specific serum IgE was used mixed panel of 36 allergens. CLIA technology is semi-quantitative, with detection by chemiluminescence and uses CLA-1 luminometer.

Data were loaded and processed using SPSS 18.0 statistical functions. Were used ANOVA, Student's t-test, correlation Kruskall-Wallis. Pearson correlation coefficient (r) gives the degree of linear association between two variables of the same group. In the graphical expression was also used and the coefficient R^2, which is the square of the Pearson coefficient.

Results, discussions

Study I. Evaluation of the relevant factors for the development of asthma and allergic inflammation markers in patients with asthma.

From the demographic data presented in the personal study, it is clear that the age of the patients included in the study ranged from 1 to 14 years, 77.9% of patients with the age between 1 - 4 years. The results are similar to those found in the specialty literature where is reported the beginning of asthma in the age of 5 years. Despite the clinical exploration and difficulties that may be criminalized in diagnosing asthma in young children, over 80% of the asthma cases are diagnosed at a preschool age. Increased prevalence of early age was recorded in ISAAC study Phase 3, where there was observed an increased incidence of respiratory allergies in the age group 6-7 years (3).

Seasonal distribution located the increased frequency of admissions in autumn (30.4%), in spring (28.1%) and in winter (25.2%), due to the fact that certain environmental factors have different seasonal activities. Seasonal variations in asthma have been demonstrated, the disease having the most increased incidence in September. In spring there is a decrease in immune status and a cluster of airborne allergens and in autumn due to return to the collectivity appear more cases of asthmatic respiratory disorders (4). Allergic asthma is often seasonal and is observed mainly in children and young adults. The unseasonal form can result in feather allergy, in animal brushes, in dust mites and in fungi and other antigens commonly present in the environment.

Regarding the declarative exposure to pneumo-allergens, from this study shows that the exposure to house dust prevailed in all subgroups of the study, about 40% of children having contact with at least one of the allergens *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*. There were no significant differences between genders. These results complete the data available in the literature that have shown that exposure during early childhood in house dust is involved in the development of asthma, being registered a relative risk 5^{th} time increased in those exposed (5). In the present study, there was an increased exposure of children to mold (20%), another important allergen. Literature suggests that after exposure to mold, the symptoms can be aggravated can also be triggered asthmatic exacerbation. Fungal spores are responsible both of seasonal forms and also of perennial respiratory allergies (6). Regarding exposure to animal dander, in the lot of the personal study, it was found a frequency of 31.3% in children that come from rural areas and 15% for those in urban areas ($p = 0.031$). The role of exposure and awareness to animal dander is uncertain. The studies are controversial, some showing that exposure to these allergens leads to an increased risk of developing asthma, while others argue that it is a beneficial exposure (7).

In the present study, the processed data showed that wheezing and asthma diagnosis was more common in male children (55.9%), the sex ratio being of 1.27: 1. Taking into account the age of the population included in the present study, the obtained results coincide with the data available in the specialty literature. The prevalence of asthma is almost two times higher in boys than in girls up to 14 years. As children mature, the gender gap is reversed in adulthood asthma prevalence is higher in women than in men. The age at which changes the report appears to be 12 years due to the rising incidence of respiratory allergic diseases in girls in the 2^{nd}age decade. Several explanations have been proposed: the small

diameter of airways compared with the lung volume in boys and the immunological differences between the genders could be the cause of this report (8).

Data analysis revealed that 88.2% of patients included in the personal study, came from urban areas. These results are similar to literature data claiming that allergic diseases are more common in urban than in rural areas. Modern living has led to increased prevalence in urban areas. In numerous studies has been shown that in the rural environment is exerted a protection regarding the occurrence of allergic diseases, exposure to pneumo-allergens, contact with animals, in general microbial and allergen stimulation of child leading to a more competitive development of the immune system (9). In urban areas, increased levels of vehicle emissions have been linked to an increased incidence of allergic diseases. Air pollutants are considered risk factors, but their intervention in asthma development remains unknown. However, many researchers consider the urban environment, by air pollutants, as a higher predisposing factor compared to rural areas (10).

Within the personal study, family medical history of atopy were present in 49.3% of patients, allergic antecedents to 1st degree relatives (32.4%) being a strong predictor for the development of asthma. Currently, the literature recognizes genetic determinism in the development of allergic asthma. The risk is multiplied if there is atopy in both parents, maternal atopy remains the major risk factor for asthma (11).

The way of delivery is a predisposing factor in the development of asthma. Of all children included in my study, most were born naturally, however 27.9% were delivered by cesarean section. Recent analyzes have evaluated the relationship between kinds of birth and incidence of asthma in children. Researchers support the protective role of naturally birth, proposing possible theories, closely related to the hygiene hypothesis: early colonization by bacterial transfer from the mother, can help postnatal development of the immune system; Another possible explanation would be that hormones released during labor that may pose a protective role for the child (12).

In the personal study, 68.4% of children were breastfed and the remaining participants in the study, were fed artificial or mixed. After data analysis there has been no correlation between the type of food and diagnosis. Within the current literature are described contradictory comments regarding breastfeeding as a measure of prevention of allergic children (13).

Of the children included in the study, only 5.1% were exposed to cigarette smoke during pregnancy, frequency of exposure, was not correlated with the frequency of asthma diagnosis. In contrast, the literature states that smoking during pregnancy increases 4 times the risk of wheezing and allergic sensitization in children. It was also highlighted a possible further impairment of lung function, bronchial inflammation and thus the occurrence of asthma in childhood (14). In terms of postnatal exposure to cigarette smoke in children from rural areas tobacco smoke was present in a proportion of 30%, interpretable results, that can be explained, however, by the existence of a socioeconomic low status and the poor education of parents.

After the biological evaluation, the results revealed that, neutrophils were increased in 49.3% of cases, with a significantly higher average, in children over 6 years (6.52 *vs*. 4.89 x 10^3/ml; p = 0.05). The average values in the leukocyte formula expressed as percentage, were within normal limits, 40.4% of cases exceeded the limit of reference. In lymphocytes, the absolute value was below the minimum benchmark in 68.4% of cases; there were no significant differences in any of the subgroups of analysis. Monocyte percentage values were in the range of 1,60 to 17,11% ,in 9.6% of cases was recorded the exceeding of the maximum reference limit (1-12%). Regarding the percentage of basophils, 11.8% of all patients showed values above the reference percentage. The highest average value was noted in the patients from rural areas and in the age group under 4 years.

Individual values in the percentage of eosinophils, exceeded the reference limit (0-4%) in 56 children (41,2% of total group). On the studied cases, were observed significantly higher values of eosinophils in children from rural areas (p = 0,036), resulting in inconsistency with literature data that consider the rural protection in the development of allergies (15).A significantly higher average value of the percentage of eosinophils was recorded 4,38% in women, while in men it was 3,74% (p =0,047) values, in accordance with the literature, which considers eosinophils as a sex-dependent biomarker (16). The average values of eosinophils in patients with asthma were significantly higher compared to the values registered in patients with recurrent or occasional wheezing. Depending on the diagnosis, there has been a significant difference in the percentages of eosinophils between the recurrent and occasional wheezing (4 times higher), the observation being consistent with the literature data that, beyond the controversy, consider increasing the number of eosinophils as a marker of allergy, or parameter risk for developing allergic pathology (17).

After assessing the relationship between eosinophils and total IgE resulted that the values of the two markers were poorly correlated (r= +0,194; R^2=0,0375; p=0,042), this correlation being sometimes used in diagnosing of asthma. The two markers have registered higher in the case of asthma, and for recurrent wheezing, values consistent with the observations in the literature, which relate to the connection of asthma and its severity, and which are influenced not only by one, but by a number of risk factors (18).

Eosinophils number and IL-4 values, showed a moderate direct correlation (r= +0,670; R^2=0,4485; p=0,001) having increased values in asthmatic children. The data are consistent with the findings of other researchers (Borres et al. - increases both the number of eosinophils and also the IL-4 levels in children who developed allergic disease in the first 6 years of life) (19).

In contrast with the literature data, the values of IFNγ were week correlated directly with the values of eosinophils (r = +0.226; R2=0.0513; p=0.048). Atopic asthma is characterized in addition by a correlation between the intensity of eosinophilic inflammation and increased of IL-4 concentration with a decrease in parallel, the production of IFNγ(20).

Taking into consideration the marked inflammatory pathology, the increased levels of obtained C-reactive protein (CRP) are in agreement with the literature (21). Individual values have a wide variance in the range of 3-193 mg/L. In 31.6% of children CRP exceeds maximum reference limit (<10 mg/L). C reactive protein has recorded the most increased medium level in children with asthma (23,0 ± 7,09 mg/l) and lowest in children with occasional wheezing (11,59 ± 7,09 mg/l). The study showed the association with increased levels of CRP serum in males (14,26 vs 10,53 mg/L; p = 0,031), with the age less than 4 years (12,914 vs 10,31 mg/L; p=0,637) and with origins in urban areas (12,65 vs 12,15 mg/L; p=0,925). The correlation between C-reactive protein values and total IgE levels was direct, of moderate intensity, about 29% of children have associated increased values of total IgE and CRP (r= +0,288; R^2=0,1478; p=0,002), result which can be extrapolated to the general population. However, assessing the relationship between CRP levels and IL-4 was noted a poor indirect correlation.

The complement system (C3) plays an integral role in the host immune responses. On the studied cases, the individual values were in the range of 71-210 mg/L, 22.8% have exceeded the maximum reference limit and 4.4% the lower limit (90-180 mg/L). Increased

levels were female gender, age group over 4 years and children from rural areas. C3 serum levels were increased in children with asthma as for those with occasional wheezing, was not performed a direct correlation with the diagnosis. Specialty literature presents positive correlations between the values of C3 and asthma severity, initiation and amplification of the inflammatory response can be achieved by activating the complement cascade (22). Evaluation of the personal obtained data in this study showed a week correlation directly statically insignificant between the values of IL-4 and C 3. Some researchers have made a connection between IL-4, and the complement system, the increased levels of C3 being the result of Th2 cytokines actions involved in the pathogenesis of asthma (23)**.** Serum Complement C3 recorded the lowest average value in children with recurrent wheezing (120,70 ± 30,39 mg/l) and highest in those with asthma (177,81 ± 26,79 mg/l) (p = 0,001). Total IgE values were significantly correlated with levels of C3, 24.9% of patients with increased IgE values, that recorded increased values of serum complement (r= +0,249; R^2=0,0654; p=0,012).

Regarding protein electrophoresis, individual values of albumin percentage were in the range of 54 to 67,80%. There were no significant differences in medium values between sexes, age groups and environmental origin (p> 0,05). Of globulin, electrophoretic fraction Alpha 2 had individual values above the maximum reference percentage (5,9 to 11%) to 50.7% of children, ranging from 9,20% to 15,30%. Significant differences of medium values were noted in males (12,11% *vs* 10,56%; p=0,048) and in the age group 0-6 years (11,98 *vs* 10,85%; p=0,031). There were no significant differences in medium values of origin (11,79*vs* 12,22%; p=0,347). Individual values of the albumin/globulin report were overwhelmed the reference levels (1-2) in 11.8% children, ranging from 1,27 to 4,46.

Serum levels of immunoglobulin A and M were within the reference ranges, individual values of IgG recorded values above the maximum reference limit (400-1250 mg/dL) in 41.9% of cases. The increase in IgG, as a response to the inflammatory process, can contribute to the serological diagnosis of infections. There were not recorded significant differences between sexes, age groups and or environmental origin (p>0,05). Immunoglobulins IgA were significantly lower in patients with asthma (p = 0,001), results that consistent with observations from literature, which claim that the levels of IgA are lower in allergic individuals (24). Some authors suggest that atopic manifestations are dependent of the delayed maturation IgA production (25).In this study, 26% of children associated

increased values of IgM associated with low levels of lymphocytes, results that can be extrapolated to the general population because the correlations were statistically significant.

Regarding total IgE level in this personal study, a 36% percent of patients recorded values above the maximum limit of reference. Were noted significant medium values, increased in the age group over 4 years (70,46 *vs* 48,97 kU/L; p=0,001) and in children from urban areas (59,57 *vs* 28,81 kU/L; p=0,001). The results did not show any statistically significant correlation with the patients' gender, family history, or type of food in the first 6 months of life, similar to the opinions of the specialty literature (26).

We evaluated the relationship between total IgE levels and IL-4, which correlates moderately positive, but statistically significant (r= +0,415; R^2=0,1725; p=0,025). The conclusions are similar to those obtained in previous studies in the literature, which reported increased levels of both IgE and IL-4 in patients with asthma (20).

Individual values of total IgEwere correlated with the parameters of leukocyte formula: direct neutrophils (r= +0,258; R^2=0,0665; p=0,006), eosinophils (r= +0,194; R^2=0,0375; p=0,042) and basophils (r= +0,197; R^2=0,0387; p=0,039) and indirect lymphocytes (r= -0,263; R^2=0,0692; p=0,005).

Total IgE are increased in patients with respiratory allergies, but can be influenced by age, genetic predisposition, ethnicity, immunity and some stages of the disease. Thus, measurement of total IgE levels may have limited value as a screening test for allergic diseases (27).

Total IgE are considered biomarkers of the allergic condition, while specific IgE can be used as markers of the allergic response, especially in children, where the skin tests are difficult to achieve. Personal research shows, after evaluating specific IgE, that 87% of enrolled patients were poly-sensitized, the remaining patients being mono-sensitized.

Allergic inflammation parameters presented the following correlations with food allergen specific IgE total score:

- eosinophils –moderate direct correlation (r= +0,299; R^2=0,0894; p=0,050);
- total IgE–moderate direct correlation (r= +0,484; R^2=0,2344; p=0,035);
- IL-4 – week direct correlation (r= +0,235; R^2=0,0552; p=0,575);
- IFNγ – week indirect correlation (r= -0,159; R^2=0,0227; p=0,706).

The score of food allergens Ig E specific was increased at 16,2% of children and may be associated with increased values of IgE and IL-4.

The parameters of allergen inflammation presented the following correlations with the total IgE score, specific external allergen:

- Eosinophils – week direct correlation (r= +0,138; R^2=0,0190; p=0,640);
- Total IgE– week direct correlation (r= +0,321; R^2=0,1031; p=0,352);
- IL-4 – strong direct correlation (r= -0,997; R^2=0,9940; p=0,001);
- IFNγ – indirect week correlation (r= -0,209; R^2=0,0437; p=0,653).

The score of external allergens was increased in 15,4% of the children and may be associated with increase values of IgE and IL-4.

The parameters of allergen inflammation presented the following correlations with the total IgE score, specific external allergen:

- Eosinophils – direct moderated correlation (r= +0,543; R^2=0,2948; p=0,001);
- Total IgE– week direct correlation (r= +0,249; R^2=0,0620; p=0,138);
- IL-4 – week direct correlation (r= +0,190; R^2=0,0361; p=0,683);
- IFNγ – week indirect correlation (r= -0,117; R^2=0,0136; p=0,664).

The score of internal allergens was increased to 14.7% of children and was associated with increased values of eosinophils > 1,20% (AUC = 0.895: IC95%: 0,797 to 0,992).

56% of patients had simultaneous awareness of indoor and outdoor allergens. In patients with sensitization to indoor allergens was a slight predominance of rural areas. But outdoor allergens presence awareness is significantly related to urban areas (p = 0,002).

The individual values of IL-4, with an ample variation (242%), were framed in the interval au 2.82-310 pg/ml. From the total of patients, 78,4% had IL-4 statically significantly increased. (p=0,004) medium value the greater being detected in patients with asthma (16,85pg/ml, p=0,609). In the studied cases are noted medium values that are slightly increased at the age group over 5 years (18,57 *vs* 16,12 pg/ml; p=0,895), and on genders the medium values of IL-4 were approximately equal (16,97 *vs* 16,66 pg/ml; p=0,98).

Individual values of IL-4 have underlined the following correlations

- Neutrophils –direct correlation week as intensity, but the result can not be extrapolated to the general population (r= +0,113; R^2=0,0128; p=0,247);
- Lymphocytes – indirect correlation week as intensity, but the result can not be extrapolated to the general population (r= -0,214; R^2=0,046; p=0,296);
- Monocytes – independent parameters (r= -0,102; R^2=0,0105; p=0,745);
- Eosinophils – direct moderate correlation (r= +0,670; R^2=0,4485; p=0,001);
- Basophils – indirect week correlation (r= -0,130; R^2=0,0169; p=0,562).

The direct correlation between the values of IL-4 and eosinophil values even if is moderated, is statistically significant, relationship corresponding to general observations in the literature (28).

IL-4 has made the following correlations with biomarkers that show significant differences depending on the epidemiological characteristics:

- protein C reactive – week indirect correlation (r= -0,137; R^2=0,0187; p=0,497);
- serum complement – week direct correlation (r= +0,141; R^2=0,0198; p=0,362);
- calcium – independent correlation (r= -0,007; R^2=0,00005; p=0,976);
- magnesium – week indirect correlation (r= -0,151; R^2=0,0227; p=0,538).

Analysing the relationship between the levels of total IgE and IL-4, Pearson correlation shows a moderate direct correlation (r= +0,415; R^2=0,1725; p=0,025), statistically significant, correlation that is in accordance with data of specialized literature (29).

Individual values of IFNγ with a wide variance (164%) were in the range of 1,20 to 40,80 pg/ml. On the studied cases it is observed slightly lower average values in girls (2,90 *vs* 4,24 pg/ml; p = 0,511) and in the group under 5 years (3,35 *vs* 3,90 pg/ml; p = 0,799).

Individual values of IFNγ correlated with the markers of leukocyte formula revealed the following:

- neutrophils – indirect correlation moderated as intensity, statistically significant (r= -0,412; R^2=0,1698; p=0,048);
- lymphocytes – direct correlation moderated as intensity, statistically significant (r= +0,560; R^2=0,3136; p=0,028);

- monocytes – independent parameters (r= +0,0028; R^2=0,00000005; p=0,999);
- eosinophils – week direct correlation (r= +0,226; R^2=0,0513; p=0,048);
- basophiles – week direct correlation (r= +0,196; R^2=0,0385; p=0,332).

Evaluating the relationship with the biomarkers, that presented significant differences according to the epidemiological characteristics, IFNγ presented the following correlations:

- protein C reactive – week indirect correlation (r= -0,161; R^2=0,026; p=0,497);
- serum complement – independent parameters (r= -0,108; R^2=0,0117; p=0,362);
- calcium –independent parameters (r= -0,06; R^2=0,0036; p=0,976);
- magnesium – week independent correlation (r= -0,103; R^2=0,0106; p=0,538).

Analyzing the relationship between IFNγ and total IgE, Pearson correlation shows a very weak direct correlation (r= +0,036; R^2=0,0013; p=0,673), statistically insignificant.

In the personal study, serum levels of IL-4 and IFNγ were above the detection limit in all diagnostic subgroups. The medium values of IL-4 were significantly increased in asthma while subgroups with wheezing diagnosis were lower. In contrast the levels of IFNγ were low in patients diagnosed with asthma. The two parameters were indirect correlated, the results being consistent with the observations from other studies (30, 31) The present study revealed a weak direct correlation between eosinophils and the levels of IFNγ, statistically insignificant.

Results, discussions

Study II. Evaluation of Interleukin-4 (IL-4) dynamics, Interferon gamma (IFNγ) Serum eosinophils and total IgE in these patients after treatment with probiotics;

After four weeks of treatment, there was a decrease of eosinophils (expressed as percentage), statistical significant (p = 0,05). At the beginning of the treatment, there was a medium value of 4.03% eosinophils, this value decreased after a month, with 2 % to the value of 2,03%.

The decrease in total IgE levels was significantly correlated with the decrease in the number of eosinophils (p = 0,05). A decrease in the medium valued of eosinophils, respectively total IgE, was recorded, observing larger differences in males. Among age groups

was not underlinedany significant decrease of eosinophils number (-3,82*vs* -1,02%; p = 0,08) and total IgE levels.

When assessing patients after four weeks of treatment it is noted a statistically significant decrease in serum levels of IL-4. Thus, at the beginning of the treatment, there has been recorded a medium value of IL-4 to 16,85pg/ml, this value decreased during the treatment to 6,44 pg/ml, statistically significant decrease (p = 0,005).

The evolution of IL-4 and IFNγ is indirectly correlated, the medium values of IL-4 decreases by 10,4pg/ml, while that of IFNγ increases by 2,42 g/ml. According to the literature data, the treatment with probiotics increase the levels of IFNγ in parallel with the decrease in IL-4 levels, such effects are dependent on the used strain, indicating a change in the balance of IFNγ and IL-4 (32).

In many clinical studies was underlined the probiotic potential that to induce the release ofcytokine Th1, and alsoIFNγ (33) and to inhibit the production of cytokines Th2, and also IL-4 (34, 35). Although, this balance change Th1/Th2 after the treatment with probiotics is controversial (36,[37]).

In genders was underlined decreases of values IL-4 (-17,08*vs* -12,62 pg/ml; p=0,697) and increases of the values IFNγ (2,38 *vs* 2,47 ng/ml; p=0,963) greater at the males. Analog, by age, were highlighted decreases of the values IL-4 (-16,17*vs* -13,61 pg/ml; p=0,832) and increased of the values IFNγ (3,15 *vs* 0,69; p=0,208).

Decreaseof values IL-4 and total IgE is, in both cases, higher in patients with recurrent wheezing compared with those with asthma, the lowest decrease being recorded in patients with occasional wheezing, differences between the initial values and those recorded after the administration of probiotics is significantly higher in these situations.

From a statistical point of view, the analysis of medium values of allergic inflammation markers post-treatment concluded that the highest values of IgE, eosinophils and IL-4 are found in children with asthma (p <0,05), while in children with occasional wheezing were noted the highest medium values of IFNγ (p = 0.001).

If at the beginning of the study were noted significant correlations, direct between the values ofIgEand the number of eosinophils(r= +0,192; p=0,042), between the levels of IgE and IL-4 (r= +0,415; p=0,025), between the number ofeosinophils and the values IL-4 (r= +0,670; p=0,001) and indirect between the values of eosinophils and the values of IFNγ (r= -

0,226; p=0,048), at the end of the study, the correlations were not statistical significant. Correlation between decreased number of peripheral eosinophils and total IgE levels was direct, statistically significant, but moderate in intensity (r= +0,278; R^2=0,0775; p=0,012).

The correlation between the changes of IFNγ and IL-4 levels was indirect, moderate in intensity, being underlined the fact that 25.6% of patients associated the increase of IFNγ values with the decrease of IL-4 levels (r= -0,256; R^2=0,0653; p=0,022).

Cytokines report value IFNγ/IL-4 initial, ranged from 0,006 in patients with occasional wheezing up to 5,13 in those with recurrent wheezing, without being recorded significant differences between the medium values on the basis of the diagnosis (p = 0,201). The highest average ratio value IFNγ/IL-4 was observed in patients with recurrent wheezing (0,753 *vs* 0.389 occasional wheezing and respectively 0,465 asthma).

After the treatment, the report of cytokine values IFNγ/IL-4 varied from 0,03 in patients with asthma up to 26,88 in those with recurrent wheezing, being registered significant differences of medium values depending on the diagnosis (p=0,027). The great medium values of the IFNγ/IL-4 report were underlined in patients with wheezing (2,001 occasional wheezing *vs* 2,893 recurrent wheezing and respectively 0,744 asthma).

Conclusions

- The most common diagnosis was that of asthma (39.7%), followed by recurrent wheezing (38.7%).
- Patients included in the study were males, aged between 1-4 years. The maximum frequency of admissions indicates a seasonal distribution mainly in cold seasons. Regarding the declarative exposure to different pneumo - allergens, the house dust was dominant in all subgroups.
- There was no corelations between diagnostic severity and the way of delivery, pre and post natal exposure to cigarette smoke and natural or formula feeding.

The biological evaluation revealed that:

- Blood eosinophilia was observed in 41,2% of cases, the number of eosinophils is correlated with the severity of the diagnosis. There were noted significantly higher

values of eosinophils in children from rural areas (p = 0,036), in feminin gender (p=0,047) and in asthma diagnostic (p=0,001).

- The correlation established between the high values of eosinophils and total IgE, was weak (r=+0,194; R^2=0,0375; p=0,042). The correlation established between the eosinophils and total IL-4 levels, was high (r=+0,670; R^2=0,4485; p=0,001).
- Increased levels of obtained C-reactive protein, positively correlated with the severity of the diagnosis, the male gender, respectively urban areas (according to literature). The correlation between C-reactive protein values and the levels of total IgE levels was direct, moderate, and within the relationship between levels of IL-4 and C-reactive protein was noted a week indirect correlation.
- Decreasing IgA may be correlated with asthmatic status (p=0,001)
- Increased of serum levels IgEwas highlighted in 36% of patients, mostly on children over 5 years age and from the urban areas.
- Total IgE levels were correlated significantly positive and statistical significant (p = 0,001), both to the values of IL-4, and to the values of eosinophils.
- Analysing the relationship between the values of IFNγ and total IgE, respectively the values of IFNγ and the levels of eosinophils, Pearson highlights in both cases a weak direct correlation (p=0,673, respectively 0,048), statistically insignificant, results different from those in the specialty literature.
- The medium values of IL-4 were significantly increased in asthma while subgroups with diagnosis of wheezing were lower. In contrast with the levels of IFNγ decreased in patients diagnosed with asthma. The two parameters were indirectly correlated, the results being compatible with the observations from other studies.
- Personal research suggests that after evaluating specific IgE, a percentage of 87% of the enrolled patients were poly-sensitized.

After four weeks of treatment reevaluation of immunological profile, revealed that:

- Decrease of eosinophils (expressed as percentage), was statistical significant (p = 0,05). At the beginning of the treatment, there was amedium value of 4,03% eosinophils, this value decreased after a month, with 2% to the value of 2,03%.

- The medium values of total IgE decreased by 26,66 kU/l. The decrease in total IgE levels was significantly correlated with the decrease in the number of eosinophils (p = 0,05).
- At the beginning of the treatment, there has been recorded a medium value of IL-4 to 16,85pg/ml, this value decreased duringthe treatment to 6,44 pg/ml, statistically significant (p=0,005).
- The evolution of IL-4 and IFNγ is indirectly correlated, the medium values of IL-4 decreases by 10,41 pg/ml, while that of IFNγ increases by 2,42 g/ml. According to the literature data, the treatment with probiotics increase the levels of IFNγ in parallel with the decrease in IL-4 levels, indicating a change in the balance of IFNγ and IL-4.

Selective bibliography

1 Fiocchi A, Pawankar R, Cuello-Garcia C, Ahn K, Al-Hammadi S, Agarwal A et al. World Allergy Organization-McMaster University Guidelines for Allergic Disease Prevention (GLAD-P): Probiotics. World Allergy Organization Journal. 2015;8(1):4.

2 Bacharier et al. European Pediatric Asthma Group. Diagnosis and treatment of asthma în childhood: a Practall consensus report. Allergy 2008;63(1):5-34.

3 Asher MI, Montefort S, Bjorksten B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006;368:733–743.

4 Randolph C. Seasonality of asthma: a retrospective population study. Pediatrics. 2014;134 Suppl 3:165-6.

5 Celedon JC, Milton DK, Ramsey CD, et al. Exposure to dust mite allergen and endotoxin in early life and asthma and atopy in childhood. J Allergy Clin Immunol 2007 Jul;120(1):144–149.

6 Sachin N. Baxi, MD1 and Wanda Phipatanakul. The Role of Allergen Exposure and Avoidance in Asthma MD, MS1 Adolesc Med State Art Rev. 2010 April ; 21(1): 57–ix.

7 Mandhane PJ, Sears MR, Poulton R, et al. Cats and dogs and the risk of atopy in childhood and adulthood. J Allergy Clin Immunol 2009 Oct;124(4):745–750. e744.

8 From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2015. Available from: http://www.ginasthma.org/.

9 N. Nicolaou, N. Siddique, A. Custovic Allergic disease in urban and rural populations: Increasing prevalence with increasing urbanization Allergy 2005; 60(11): 1357–1360.

10 Adler A, Tager I, Quintero DR. Decreased prevalence of asthma among farm reared children compared with those who are rural but not farm-reared. J Allergy Clin Immunol 2005;115:67–73

11 Bjerg A, Hedman L, Perzanowski M, Platts-Mills T, Lundback B, Ronmark E. Family History of Asthma and Atopy: In-depth Analyses of the Impact on Asthma and Wheeze in 7- to 8-Year-Old Children. Pediatrics. 2007;120(4):741-748.

12 Josef Neu, MD, Jona Rushing, MD.Cesarean versus Vaginal Delivery: Long term infant outcomes and the Hygiene Hypothesis. Clin Perinatol. 2011 June ; 38(2): 321–331.

13 Kull I, Melen E, Alm J, Hallberg J, Svartengren M, van Hage M et al. Breast-feeding in

relation to asthma, lung function, and sensitization in young schoolchildren. Journal of Allergy and Clinical Immunology. 2010;125(5):1013-1019.

14 V. Fuentes-Leonarte et al.Pre- and postnatal exposure to tobacco smoke and respiratory outcomes during the first year Indoor Air 2015; 25: 4–12

15 Priftis K, Mantzouranis E, Anthracopoulos M. Asthma Symptoms and Airway Narrowing in Children Growing up in an Urban versus Rural Environment. J Asthma. 2009;46(3):244-251.

16 Rubner F, Jackson D, Tisler C, Rajamanickam V, Gern J, Lemanske R. Relationships Among Eosinophils, Asthma, and Sex in a High-Risk Birth Cohort. Journal of Allergy and Clinical Immunology. 2015;135(2):AB228.

17 Oymar K, Havnen J, Halvorsen T, Bjerknes R. Eosinophil counts and urinary eosinophil protein X in children hospitalized for wheezing during the first year of life: prediction of recurrent wheezing. Acta Paediatr. 2001;90(8):843-9.

18 Siroux V, Oryszczyn MP, Paty E, Kauffmann F, Pison C, Vervloet D, Pin I. Relationships of allergic sensitization, total immunoglobulin E and blood eosinophils to asthma severity in children of the EGEA Study. Clin Exp Allergy. 2003;33(6):746-51.

19 Borres MP, Björkstén B. Peripheral blood eosinophils and IL-4 in infancy in relation to the appearance of allergic disease during the first 6 years of life. Pediatr Allergy Immunol. 2004;15(3):216-20.

20 Bettiol et al. Cytokine production from peripheral whole blood in atopic and nonatopic asthmatics: relationship with blood and sputum eosinophilia and serum IgE levels. Allergy. 2000;55(12):1134-41.

21 Balakrishnan M, Gaki N, Vikas D, Charanjeet K. C reactive protein as a marker of asthma control. International Journal of Basic and Applied Medical Sciences 2013;3(3):114-119.

22 Fattah M, El Baz M, Sherif A, Adel A. Complement components (C3, C4) as inflammatory markers in asthma. Indian J Pediatr. 2010;77(7):771-773.

23 Mosca T, Menezes MC, Dionigi PC, Stirbulov R, Forte WC. C3 and C4 complement system components as biomarkers in the intermittent atopic asthma diagnosis. J Pediatr (Rio J). 2011;87(6):512-6.

24 Grundbacher FJ, Massie FS. Levels of immunoglobulin G, M, A, andE at various ages in allergic and nonallergic black and white individuals. J Allergy Clin Immunol. 1985 Jun;75(6):651-8.

25 Lúdvíksson BR, Eiríksson TH, Ardal B, Sigfússon A, Valdimarsson H. Correlation between serum immunoglobulin A concentrations and allergic manifestations in infants. J Pediatr. 1992;121(1):23-7.

26 Satwani H, Rehman A, Ashraf S, Anwar H. Is Serum Total IgE levels a good predictor of Allergies in Children? 2009;59(10).

27 Huang H-WL, Ko-Huang W., Ruey-Hong S., Hailun S., Jinan L., Ko-Hsiu. Distribution of allergens in children with different atopic disorders in central Taiwan. Acta Paediatr. Taiwan, 2006;47(3):127-134.

28 Possa S, Leick E, Prado C, Martins M, Tibério I. Eosinophilic Inflammation in Allergic Asthma. Frontiers in Pharmacology. 2013;4-7.

29 Mishra JK et al. An Immunological Study of Bronchial Asthma with Special Reference to Interleukin-4, Interferon-γ and Immunoglobulin E Indian J Allergy Asthma Immunol 2011; 25(2): 91-96

30 Bogic M et al. Clinical significance of measurement of interleukin 4 and interleukin 5 serum concentrations in bronchial asthma. Jugoslov Med Biochem 2004;23:51-54

31 Shahid SK, Kharitonov SA, Wilson NM, Bush A, Barnes PJ. Increased interlukin-4 and decreased interferon-c in exhaled breath condensate of children with asthma. Am J Respir Crit Care Med 2002; 165: 1290-3

32 Sashihara MT, Sueki N, Ikegami S. An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. J. Dairy Sci 2006;89:2846-2855.

33 Ghadimi, D., Fölster-Holst, R., De Vrese, M., Winkler, P., Heller, K.J., Schrezenmeir J. Effects of probiotic bacteria and their genomic DNA on T H1/TH2-cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. Immunobiology 2008;213:677-692.

34 Chen YS, Jan RL, Lin YL, Chen HH, Wang JY. Randomized placebo-controlled trial of lactobacillus on asthmatic children with allergic rhinitis. Pediatr. Pulmonol 2010;45:1111-1120.

35 Kawase, M., et al. Effect of fermented milk prepared with two probiotic strains on Japanese cedar pollinosis in a double-blind placebo-controlled clinical study. Int. J. Food Microbiol 2009;128:429-434

36 Tamura M, et al. Effects of probiotics on allergic rhinitis induced by Japanese cedar pollen: randomized double-blind, placebo-controlled clinical trial. Int. Arch. Allergy Immunol

2007;143:75-79.

37 Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. Journal of Leukocyte Biology. 2011;89(5):685-695.