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UPDATE ON ALLERGIC DISEASES IN CHILDHOOD

CONTENTS

TRYPTASE AND HISTAMINE MAY SUPPORT ORAL FOOD CHALLENGE IN THE DIAGNOSIS OF ALLERGY

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Allergen-specific immunoglobulin E (IgE) reactions lead to acute degranulation of mast cells and basophils and release of stored mediators, particularly tryptase and histamine, which can be measured in vitro after reactions. The aim of this study was to investigate the utility of serum tryptase and plasma histamine during oral food challenge (OFC) in 103 children with suspected food allergy, in order to support the diagnosis of a IgE-mediated reaction. Blood samples for serum tryptase and plasma histamine were collected before the OFC and after the onset of allergic symptoms or after 60 minutes from test completion. Serum tryptase and plasma histamine were measured by a fluoroenzyme immunoassay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) according to the manufacturer's instructions. A correlation between serum tryptase and plasma histamine distributions was observed after OFC ($p = 0,0035$). A correlation was also observed for both serum tryptase and plasma histamine before and after OFC ($p < 0,0001$). Subjects with positive response to OFC had significantly higher values ($p = 0,0375$) of serum tryptase compared to subjects with negative response. The plasma histamine distribution showed a significant difference between measurements before and after OFC, both in the complete population ($p < 0,0001$), and considering the response (negative OFC: $p < 0,0001$; positive OFC: $p = 0,0181$). The diagnostic work-up of IgE-mediated food allergy may include determination of serum tryptase and plasma histamine, in order to support the results of OFC. These markers are strongly related to the same IgE-mediated mechanism and, as they can be both easily measured, can confirm the allergic nature of a reaction in the real-life setting of food allergy.

The oral food challenge (OFC) actually represents the gold standard investigation for the objective diagnosis of IgE-mediated allergy (1). OFC must be performed in a specialist setting with emergency support immediately available, due to the risks of severe reactions, including anaphylaxis (1).

Immediate allergic reactions proceed through allergen-specific immunoglobulin E (IgE) binding, leading to acute degranulation of mast cells and basophils and release of stored mediators, particularly tryptase and histamine, which can be measured in vitro after reactions (2). In this setting, serum total tryptase levels should be measured from 15 minutes to 3 hours after symptom onset, while plasma histamine peaks at 10 minutes after symptom onset and disappears in 60 minutes (3).

Serum tryptase has been proposed for use as a marker of anaphylaxis, and it has proven useful for validating drug and insect sting-induced anaphylactic reactions, but not yet for food-induced anaphylaxis (4); however, a supportive diagnostic role has been recently proposed for food-induced anaphylactic reactions in children (5). Moreover, serum basal tryptase levels has been recently showed to be predictive of moderate to severe anaphylaxis in children with food allergy (6).

The role of histamine in immediate allergic reactions is well known, where it acts as the most

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important mediator which produces the majority of signs and symptoms in these reactions. Some studies suggest that levels of plasma histamine are correlated with the severity of the reaction (7). During an allergic reaction, histamine is released from the mast cells and basophils in parallel with tryptase (8).

The aim of this study was to investigate the utility of serum tryptase and plasma histamine during OFC in order to support the diagnosis of food allergy in children.

MATERIALS AND METHODS

Study Population

One hundred and three patients (35 females, 68 males; mean age: 4,5631 years, age range: 0-16 years) were evaluated for food allergy at the Foundation IRCCS Policlinico San Matteo of Pavia from August 2011 to May 2014. Some patients had sensitizations to inhalants, food, egg or milk. In particular: 36 were mono-sensitized to one of these allergens (5,83% to inhalants, 14,56% to food, 6,8% to egg and 7,77% to milk) and 55 poli-sensitized to two or more allergens (37,86% to inhalants and food, 5,83% to egg and milk, 2,91% to inhalants and milk and 3,88% to inhalants and egg). Only 12 patients did not present any sensitization.

Some patients also presented underlying diseases such as asthma, rhinitis, wheezing, atopic dermatitis, urticaria, skin rash, or failure to thrive. In particular, 55 subjects presented with one underlying disease and 17 had more than one. The remaining 31 patients did not show any disease. Subjects with underlying diseases have been divided into 3 categories: respiratory (16 patients), cutaneous (47 patients) and mixed (9 patients).

Procedures and Measurements

An open OFC was performed for all patients, after obtaining informed consent from parents. OFC was performed for milk (43 patients), egg (51 patients), tree nuts (8 patients) and rice (1 patient). In order to avoid severe reactions, patients received the food in titrated doses, which range from 3 mg to 3 g, and at set intervals, according to validated guidelines (1). OFCs were stopped as soon as objective clinical reactions were observed (positive response) or the last dose was consumed without clinical symptoms (negative response).

Basal levels of specific IgE were available for all patients. Specific serum IgE levels were measured by immunofluorometric assay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) and expressed in kU/L. Specific IgE levels higher than 0.35 kU/L were considered positive. Blood samples for serum tryptase and plasma histamine were collected before the OFC and after the onset of allergic symptoms or after 60 minutes from test completion. Serum tryptase and plasma histamine were measured by a fluoroenzyme immunoassay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) according to the manufacturer's instructions. The cut-off for tryptase is 3,8 µg/l (pathologic value > 11,4 µg/l), while for histamine is 1 ng/ml.

Statistical analysis

Statistical analysis was performed using the statistical software package Medcalc 9 (Frank Schoonjans, BE). Medians and percentiles (25th and 75th, IQR) were used as descriptive statistics, due to non-normal distribution of the variables. The correlations between tryptase and histamine and between the distributions of each parameter before and after OFC have been evaluated. Only one outlier in histamine distribution has been detected and excluded from the analysis. This value for histamine before OFC was 7,5 times the standard deviation and for histamine after OFC it was 7,78 times the standard deviation. The non-parametric Mann-Whitney's test was used to compare samples resulting positive or negative to OFC. The non-parametric Wilcoxon's test for paired samples was used to compare values before and after OFC. In addition the non-parametric Kruskall Wallis test for analysis of variance was used to compare more than two samples according to sensitization and pathologies. Chi-square's test was used to study the relationship between sensitization and OFC. In addition logistic and linear multiple regression analysis was performed, respectively, to correlate and predict the response to OFC, and to predict histamine and tryptase after OFC. The stepwise strategy was adopted to select significant

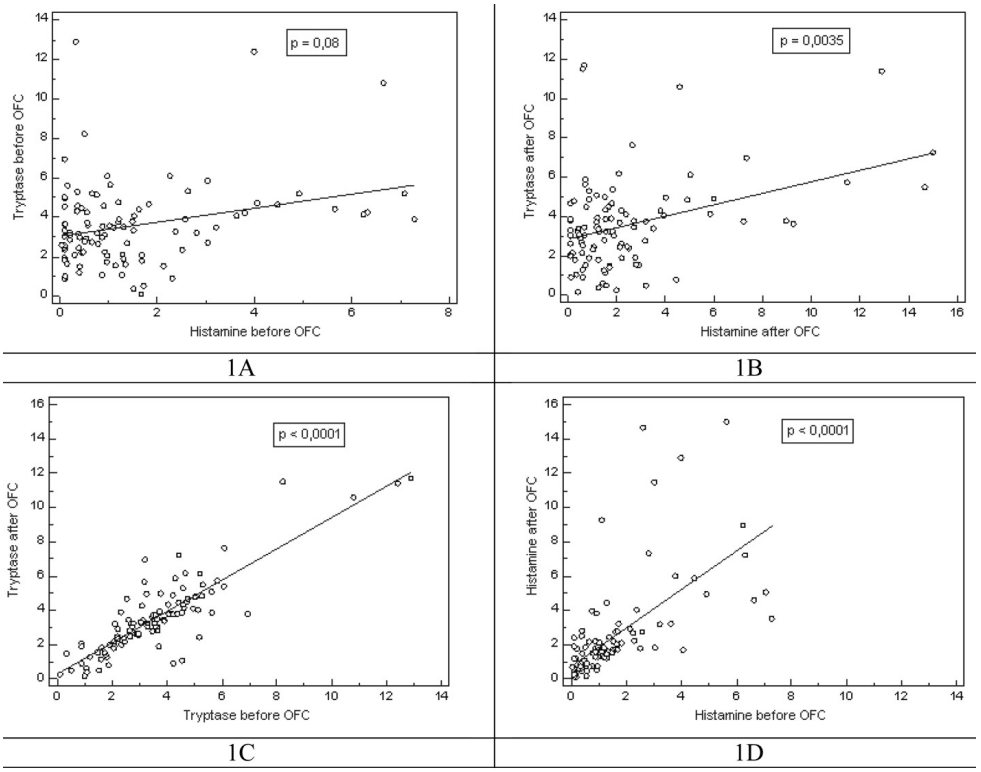

Fig. 1. *Correlation between distributions was evaluated: correlation between tryptase and histamine before OFC (Fig. 1A) and after OFC (Fig. 1B). There was a correlation between tryptase before and after OFC (Figure 1C) and between histamine before and after OFC (Fig. 1D).*

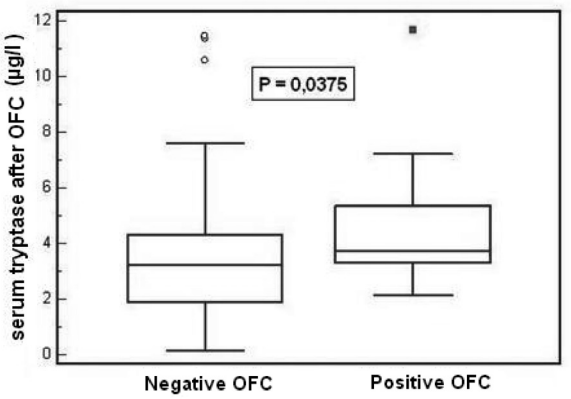

Fig. 2. *Tryptase measured after OFC. Values corresponding to a positive response were higher than negative ones.*

independent predictors. Values of $P \leq 0.05$ were considered to indicate statistical significance.

RESULTS

After OFCs, mild allergic reactions (positive response) were documented in 15 patients; no symptoms after exposure of the challenged food (negative response) were observed in 88 patients. In particular, OFC for milk was positive for 4 patients; 2 of these were also positive to the major milk allergen (mean = 0,62 kU/L). OFC for egg was positive for 9 patients and 8 of them were positive to the major egg allergen (mean = 5,246 kU/L). All patients with positive response developed symptoms during the challenge or immediately after the test completion; in addition, no anaphylaxis was observed. In this group, 10 patients presented with cutaneous

Table I. *Comparison of Tryptase and Histamine values between negative and positive response groups, stratified according to pre- and post-OFC measures (Mann Whitney Test).*

Tryptase		**Pre OFC**			**Post OFC**		
	n	median	25^{th}-75^{th}	p	median	25^{th}-75^{th}	p
Negative OFC	88	3.23	2.095 – 4.54		3.22	1.88 – 4.315	
Positive OFC	15	3.50	2.74 – 4.38		3.74	3.29 – 5.36	
				0.3286			0.0375
Histamine		**Pre OFC**			**Post OFC**		
	n	median	25^{th}-75^{th}	p	median	25^{th}-75^{th}	p
Negative OFC	88	0.87	0.36 – 1.68		1.55	0.645 – 2.57	
Positive OFC	15	1.31	0.307 – 2.77		1.57	0.67 – 7.305	
				0.5306			0.2822

Table II. *Comparison of Tryptase and Histamine values before and after OFC, stratified according to OFC response (Wilcoxon Test for paired data).*

Tryptase				**Negative OFC**			**Positive OFC**		
	n	median	p	n	median	p	n	median	p
Pre-OFC	103	3.31		88	3.23		15	3.50	
Post-OFC	103	3.24		88	3.22		15	3.74	
			0.2301			0.0992			0.4543
Histamine				**Negative OFC**			**Positive OFC**		
	n	median	p	n	median	p	n	median	p
Pre-OFC	103	0.92		88	0.875		15	1.31	
Post-OFC	103	1.55		88	1.55		15	1.57	
			< 0.0001			< 0.0001			0.0181

involvement (urticaria and angioedema), while 5 patients had gastrointestinal symptoms (vomiting and abdominal pain).

Serum tryptase and plasma histamine distributions were evaluated before and after OFC across the complete population, and then considering responses to OFC. Serum tryptase showed the following values for median and percentiles: 3,31 µg/l (IQR: 2,21 - 4,51) before OFC and 3,24 µg/l (IQR: 2,065 - 4,432) after OFC; plasma histamine distributions showed the following values: 0,92µg/l (IQR: 0,35 – 1,71) before OFC and 1,55µg/l (IQR: 0,67 – 2,72) after OFC. A correlation between serum tryptase and plasma histamine distributions was observed after OFC (p = 0,0035), but not before (p = 0,08). Considering distributions of each parameter, a correlation was observed for both serum tryptase and plasma histamine before and after OFC (p < 0,0001) (Fig. 1).

Then, distributions were evaluated considering the response to OFC. Subjects with positive response had significantly higher values (p = 0,0375) of serum tryptase compared to subjects with negative response (Fig.2). For the other distributions, no statistically significant difference was observed (Table I).

The plasma histamine distribution showed a

Table III. *Logistic regression model for the response to OFC (positive or negative).*

Dependent variable: response to OFC				
Indipendent variables	Coefficient	Std. error	t	p
Constant	0.0336			
Tryptase after OFC	0.03168	0,0155	2.044	0.0436
Coefficient of determination R^2= 0,0397				
Significance level: P = 0.044				

Histamine and typtase before OFC, histamine after OFC, age, sex, sensitizations, pathologies and specific IgE were not included in the final model because they did not achieve statistical significance as predictors.

Table IV. *Multiple regression model to predict histamine and tryptase values measured after OFC.*

Dependent variable: Tryptase After OFC				
Indipendentvariables	Coefficient	Std. error	t	p
Constant	-0.6556			
Tryptase before OFC	0.9421	0.05214	18.068	<0.0001
Pathologies	0.5853	0.2391	2.448	0.0161
Specific IgE	0.5862	0.2357	2.487	0.0146
Coefficient of determination R^2= 0.7757				
Significancelevel: P <0.001				

Histamine before OFC. Age, sex and sensitization are not included in the model because they are not predictors.

Dependent variable: HistamineAfter OFC				
Indipendentvariables	Coefficient	Std. error	t	p
Constant	0.1821			
Histamine pre-OFC	1.5495	0.09510	16.293	<0.0001
Coefficient of determination R^2= 0.7244				
Significancelevel: p <0,001				

Tryptase before OFC, age, sex, sensitization, pathologies and specific IgE were not included in the final model because they did not achieve statistical significance as predictors.

significant difference between measurements before and after OFC, both in the complete population (p < 0,0001), and considering the response (Negative OFC: p < 0,0001; Positive OFC: p = 0,0181) (Table II).

The study population was also divided, according to sensitization, into 3 groups: no sensitized, mono-sensitized and poli-sensitized. We analyzed how the sensitization was distributed according to the response to OFC. All patients with a positive response were sensitized, but also the majority of patients with negative OFC were sensitized. No statistical significance was observed (p = 0,13).

The study population was also divided, considering the presence or the absence of underlying diseases, into 3 groups: patients without any disease, with only one disease and with two or more diseases. Only for the serum tryptase evaluated before OFC, a statistical difference was observed (p = 0.0188).

Multiple regression analyses were performed with two different aims. The first one is purely descriptive of the response, so that independent variables were

both pre- and post-test variables. The second one is a predictive aim, so that only pre-test variables were considered as predictors. In both models we also considered age, sex, the presence or absence of sensitization and underlying diseases, and the result (positive or negative) of specific IgE as independent variables. In the descriptive multivariate regression, our results showed that only serum tryptase after OFC was statistically significant to determine OFC response (Table III). In the predictive model, our results showed that none of the variables were predictors for the response to OFC.

Another multiple regression analysis was performed to predict serum tryptase and plasma histamine after OFC from their basal values, the presence or the absence of sensitization and underlying disease, age, sex and the result of specific IgEs (positive or negative). For both markers, their distributions evaluated before OFC were statistically significant. For serum tryptase regression model, also the presence or the absence of underlying diseases and the result of specific IgEs were statistically significant (Table IV).

In addition, we considered the subgroups of underlying diseases as another predictor. None of diseases' subgroups were predictors for serum tryptase after OFC, while for histamine the cutaneous group has resulted significant (p = 0,0127).

DISCUSSION

To date, in our knowledge, this is the first study monitoring both serum tryptase and plasma histamine in the diagnostic work-up of food allergy in children. A similar previous study focalized on serum tryptase and eosinophil cationic protein as additional diagnostic tools in adult patients with clinical hypersensitivity to Anisakis simplex, without any significant result (9).

Monitoring markers of inflammation during allergic reactions may help to identify the different cell types involved and clarify the mechanism of these reactions. In particular, the determination of serum tryptase and plasma histamine is useful as they allow the participation of mast cells and/or basophils in the reaction to be confirmed and provide an orientation as to the probable mechanism involved (8).

Our study shows that both serum tryptase and plasma histamine production is strongly related to the same IgE-mediated mechanism and, as they can be both easily measured, can confirm the allergic nature of a reaction in the real-life setting of food allergy.

Moreover, the values of these two markers after OFC may be affected by its response. In particular, significantly higher values of serum tryptase after OFC are observed in patients with positive response, compared to those with negative ones, even if these values are far below the established pathologic cut-off. No anaphylactic reactions were observed in the positive response group indeed. This result is apparently in contrast with previous studies, which reported a lack of tryptase elevation in food-induced anaphylaxis, which was explained as due to slow onset of reactions or because mucosal mast cells and basophils, the major players in food-induced anaphylaxis, contain less to no tryptase as compared with skin mast cells (10, 11). Serial measurements of tryptase levels have been reported to increase the sensitivity and specificity of the test (4); however, this approach has not yet been studied in the pediatric population.

Besides, a recent study detected a strong association of elevated basal levels of serum tryptase with anaphylaxis and severity of reactions in children with food allergy (6); this is consistent with the results of previous studies on patients with venom allergy (12). These findings increased the importance of serum basal tryptase in screening patients with allergy to determine groups at high-risk of reactions, including patients with mast cell activation syndrome (6).

In our study, higher values of plasma histamine after OFC were also observed in patients with positive response, compared to those with negative ones, even if this difference is not significant.

The presence of sensitizations and underlying diseases do not influence distributions. Subjects without any disease had higher values of serum tryptase than subjects with one or more diseases. This result could be explained by a possible use in symptomatic subjects of anti-allergic drugs, such as corticosteroids that reduce serum tryptase values.

The response to OFC seems not to be predicted

by any of the variables considered. Instead, both markers' basal levels could be predictive of the values after OFC; in addition, underlying diseases and specific IgE are predictors for tryptase after OFC. Then, in the diagnostic work-up of food allergy, testing specific IgE may have an added value in patients undergoing OFC.

The strength of our study was monitoring serum tryptase and plasma histamine to support the results of OFCs in children with suspected food allergy, which has never been analyzed before. However, the small number of positive responses could be considered a limitation for this study; further efforts are needed to investigate for a possible different outocome considering a bigger population.

CONCLUSION

The diagnostic work-up of IgE-mediated food allergy may include determination of serum tryptase and plasma histamine, in order to support the results of OFCs. These markers are strongly related and, as they can be both easily measured, can confirm the allergic nature of a reaction in the real-life setting of food allergy.

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ALMOND MILK: A POTENTIAL THERAPEUTIC WEAPON AGAINST COW'S MILK PROTEIN ALLERGY

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Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly following exposure to a given food. Cow's milk protein allergy results from an immunological reaction to one or more milk proteins. The principle key in the treatment of cow's milk protein allergy is the dietary elimination of cow's milk protein. Although hydrolyzed and elemental formulas are appropriate replacements, other milk products, including almond milk adequately integrated, could be administered. Here, in the light of encouraging results from our study, we focused on the anti-inflammatory and anti-oxidant properties of almond milk and we also believe that almond milk might be considered as a potential alternative in cow's milk protein allergy treatment.

Cow's milk protein allergy (CMPA) results from an immunological reaction to one or more milk proteins (1, 2). The immune reaction may be immunoglobulin (Ig)E mediated, non-IgE mediated, or mixed (3, 4). CMPA is the leading cause of food allergy in infants and children under 3 years of age (1, 3, 5, 6), prevalence is of approximately 2% to 7.5% in the infant population (1), of which only 60% are IgE-mediated (7, 8). CMPA can induce a diverse range of symptoms (e.g., gastrointestinal (50–60%), skin (50–60%) and respiratory tract (20–30%) of variable intensity (8). The diagnosis of CMPA is based on oral food challenge (9). The principle key in the treatment of CMPA is the dietary elimination of cow's milk protein. Generally, during breast-feeding, and in children aged 2 years or over, a substitute formula may not be necessary. In fact, mothers should be encouraged to continue breast-feeding while avoiding all milk and milk products from their own diet. In non-breastfed infants and in children under 2 years, replacement with a substitute formula is mandatory. Additionally, supplementary foods containing CMP or other unmodified animal milk proteins should be strictly avoided (3). If the first feeds with cow's-milk–based formula in a breastfed infant cause symptoms, the infant should return to exclusive breast-feeding without any elimination in the maternal diet. An elimination diet in formula-fed infants usually starts with an extensively hydrolyzed infant formula (eHF) with proven efficacy in infants with CMPA (9, 10). In infants with extremely severe or life-threatening symptoms, an amino acid formula (AAF) may be considered as the first choice (9). Although hydrolyzed and elemental formulas are appropriate replacements, other milk products, including almond milk adequately integrated, could be administered, especially in infants, older than 6 months, who do not accept the bitter taste of an eHF, or in cases in which the higher cost of an eHF is a limiting factor.

Protective role of almond in cow's milk protein allergy

Almond is a species of Prunusdulcis belonging to

Key words: cow's milk protein allergy, almond milk, therapy, children.

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the Rosaceae family. Almond fruit consists of 3 or, more accurately, 4 portions: the kernel or meat, the middle shell, the outer green shell cover or almond hull, and a thin leathery layer known as the brown skin of the meator the seed coat (11). The nutritional importance of the almond fruit is related to its kernel. Lipid, the main storage component in almond seeds, constituting over 50% of the total weight of the seeds, is located as intracellular oil bodies. Proteins comprise about 22 to 25% of the seeds, while 11 to 12% is represented by dietary fiber (12). Almond is also a good source of minerals and vitamin E, associated with promoting health, probiotic (13-15) and anti-oxidant effects (16), and reducing the risk of chronic disease (17). Almond components, able to reach the colon, can potentially provide the body with additional energy via microbial fermentation, and the production of short-chain fatty acids may have potential prebiotic functionality. Thus, the prebiotic properties of almond could be useful in CMPA patients with gastrointestinal symptoms. In fact, possible non-immunological etiologies include: 1) the direct effect of cow's milk protein on intestinal motility (18), 2) colonic bacterial dysbiosis, enhanced by cow's milk formulas (19), and 3) effects caused by the non-protein parts of cow's milk: carbohydrates and fatty acids (20). Hence, almond, showing a suitable carbon source for bacterial fermentation (21), exhibits potential properties that can be used as a novel source of prebiotics, increasing the populations of bifidobacteria, exerting antimicrobial activity and "barrier effect" by the indigenous microflora (22, 23) (Fig. 1). An imbalance between oxidative stress and antioxidant capacity may also contribute the development and progression CMPA. CMPA subjects reported oxidative stress, assessed by decreased antioxidant system and increased intestinal lipid peroxidation (24). In the light of these data, almond milk could be proposed as an alternative milk to influence disease course. In fact, almond components promote the subsequent increase in butyrate concentrations, entering into intermediary metabolism (25). Studies in vitro assessed that butyrate exercise pivotal anti-inflammatory, anti-oxidant, and anti-radical effects (17, 26-28). In fact, extracts of the whole almond seed, including the brown skin, shell, and green shell cover, show potent free radical-scavenging capacities (29, Fig. 1). These activities may be also related to the presence of flavonoids and other phenolic compounds that prevent cell injury with mechanisms involving free radical scavenging and metal

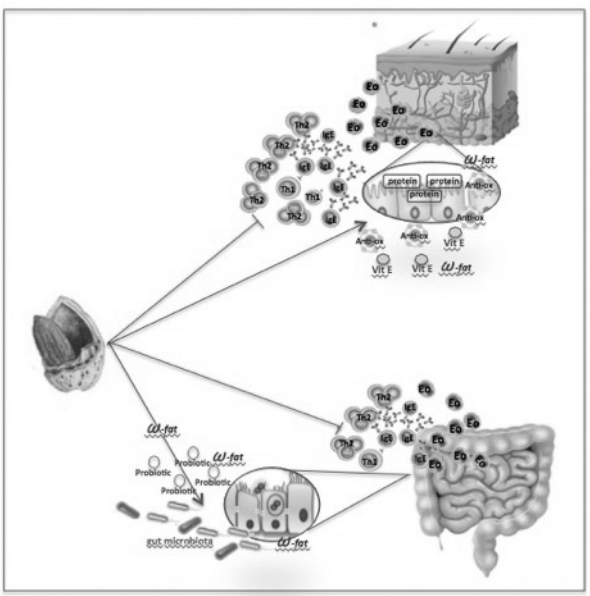

Fig. 1. *Protective role of almond milk in allergic diseases. Almond is also a good source of minerals, vitamin E, mono-unsaturated fats, proteins, and probiotics exercising anti-inflammatory and anti-oxidant effects which, in turn, reduce the risk for atopic diseases and promote good health.*

chelation (16). Moreover, in experimental model, it has been assessed that almond cell walls improved the bioaccessibility to nutrients (e.g., lipid, protein, and vitamin E) in the gut after duodenal digestion. This mechanism might also have implications for the prevention and management of allergic diseases (17, 30). Previously, authors reported encouraging results of a randomized clinical trial carried out to test the efficacy and safety of almond-milk in a group of infants with documented CMPA (31). The main efficacy outcomes were the improvement in clinical symptoms and the decrease in serum levels of soluble CD30. Additionally, no difference in growth rate (increment of weight, length and head circumference) was found, during the entire study, between infants given the almond milk and babies given the soy-based formula or the protein hydrolysate-based formula. Furthermore, conversely to supplementation with the soy-based and protein hydrolysate-based formulas, almond milk did not cause a secondary sensitization (31). In addition, although case series recently described severe malnutrition resulting from use of alternative formulation food elimination diets for atopic dermatitis (32), preliminary data, resulting from our study, reported that almond milk might be an efficacious substitute of cow milk in infants with CMPA. In fact, we evaluated dietary intake of lipids and proteins in children drinking almond milk. Precisely, we administered 2 feedings of almond milk to weaned children, older than 6 months, affected by atopic dermatitis. All enrolled patients showed normal height and weight measurements and improved clinical signs and symptoms. Due to palatability of almond milk, patients and their families also showed a good treatment adherence. Therefore, given anti-inflammatory and anti-oxidant properties of almond milk and in the light of encouraging results from our study, we believe that almond milk might be considered as a potential alternative in CMPA treatment.

CONCLUSIONS

Although hydrolyzed or elemental formulas provide a safe choice for children allergic to cow's milk, to date, alternative formula, also including almond milk, should be considered as a potential alternative in CMPA treatment. Almond milk is lower in calories than other milks. It is also free of cholesterol, saturated fat, and is naturally lactose free. Additionally, almonds are rich in nutrients including fiber, vitamin E, magnesium, selenium, manganese, zinc, potassium, iron, phosphorus, tryptophan, and copper. Thus, the abundance of these essential nutrients and trace minerals in almond milk makes it an important part of a diet. However, although almonds are also a good source of protein and calcium, almond milk is not. Therefore, almond milk should be supplemented with proteins, calcium as well as vitamin D, especially in children not weaned and/or under two years of age. The key role of almond milk in atopic children might be explained by the following properties: 1) it is an excellent source of antioxidants which protect cells from damage caused by free radicals derived from inflammatory status; 2) it suppresses pro-inflammatory responses; 3) it exhibits potential properties that can be used as a novel source of prebiotics. In the light of the above-mentioned properties, almond milk could, if adequately integrated, be an efficacious substitute of cow milk in infants with CMPA. Additionally, it might be a valid alternative especially for infants who do not accept the bitter taste of an eHF, or in cases in which the higher cost of an eHF is a limiting factor. Unfortunately, literature data are few and still controversial. Further studies are necessary in order to support almond milk as a future therapeutic strategy in CMPA patients.

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ATOPIC DERMATITIS: EXPRESSION OF IMMUNOLOGICAL IMBALANCE

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Atopic dermatitis is a chronic relapsing-remitting inflammatory skin condition, characterized by a skin barrier dysfunction resulting in epidermal damage and altered permeability to allergens and microbes. Although pathogenesis of atopic dermatitis is complex and still not fully understood, it has been hypothesized that genetic predisposition, environmental factors, and skin barrier dysfunction are involved. Innate and adaptive immune system has also a pivotal role in the development, maintenance and flare-up of atopic dermatitis. The immune-pathogenesis of atopic dermatitis is determined by the impairment of different T helper cells, of their cytokine secretion profiles as well as of their specific receptor. In this review, we focus on the current knowledge of the etiopathogenetic pathways of atopic dermatitis in relationship to the critical role of the innate and adaptive immune system, providing an unifying view.

Atopic Dermatitis (AD) is a chronic relapsing-remitting inflammatory skin condition (1-4). AD is sustained by a complex interaction between genetics, immunological, and environmental factors (5) and it is also characterized by a skin barrier dysfunction resulting in epidermal damage and altered permeability to allergens and microbes (6). Although pathogenesis of AD is complex and still not fully understood, it is well known that the immune system plays a key role in the development, maintenance and flare-up of AD (7). In fact, since the first discovery of the pivotal influence of innate and adaptive immune system in AD, several studies also assessed the role of pattern recognition receptors (PRRs) in recognizing pathogen-associated molecular pattern molecules (TLRs) (8, 9), antimicrobial peptides (e.g., cathelicidinand ß-defensin) (10, 11), and self-molecules after cell damage or death, which have recently been noted as important pro-inflammatory mediators in their role as damage-associated molecular pattern (DAMP), such as high mobility group box 1 (HMGB1) (12-16). Additionally, innate T cells are also known to play significant roles in AD (17).

Regulatory T (T-reg) cells in AD

Regulatory T (T-reg) cells play a pivotal role in immune suppression and are integral to the control of allergic or auto-antigens responses (18, 19). Transcription factor gene FoxP3 and IL-10 are responsible for the development and the function of T-reg (18, 19). AD patients have been reported to have significantly increased numbers of peripheral

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blood T-reg cells with normal immunosuppressive activity (19). Moreover, emerging evidence suggests that T-reg cells can bi-directionally convert to Th2 cells. Additionally, T-regs not only diminish Th2 immune response, but also target other cell types such as dendritic cells (DCs), basophils and eosinophils. The effects of T-regs include, among others, allergen-specific IgE regulation, and specific inhibition of mast cell degranulation (20-22).

Cytokines-Th2 in AD

The allergic cascade starts with the recognition of allergens by the antigen-presenting cells, mainly DCs, leading to Th2 polarization and switching to IgE production by B cells. The allergic response is also characterized by the synthesis of Th2 cytokines (e.g., IL-4, IL-5 and IL-13) which induce the recruitment and sensitization of effector cells including eosinophils, basophils and mast cells (23). In addition to traditional Th2 cytokines, recently discovered proinflammatory Th2 cytokines (e.g., IL-25, IL-31, IL-33 and IL-9) also participate in AD (23). Moreover, IL-4, predominantly expressed in the initial phase of acute skin inflammation, promotes Th2 cells to become refractory to Treg suppression (24). Instead, IL-13 stimulates keratinocytes to generate matrix metalloproteinase-9, which, in turn, degrades type IV collagen, a major component of the basement membrane (23). Moreover, IL-31 exercises a potent pruritogenic effect through its specific receptor expressed on cutaneous nerve fibres and dorsal root ganglia (25). IL-33 is pleiotropic cytokine, member of the IL-1 family. The synthesis of IL-33 is mediated by immunological, mechanical, and infectious triggers. IL-33 has been found to be over-expressed in the onset of AD (26).

Cytokines-Th1 in AD

Chronic phase of AD is characterized by activation of Th-1 which produce mainly IFN-γ, TNF-α, IL-8 and IL-12. In acute phase, IFN-γ, by regulating the expression of specific receptors, exercises a modulatory effect on expression of several cytokines. During chronic period, IFN-γ induces, in keratinocytes, the synthesis of IL-33 that acts on the activated T cells to further increase the release of IFN-γ, therefore contributing to drive skin inflammation towards chronic responses (27, 28). In addition, IFN-γ, by decreasing the key enzymes required for the synthesis of ultra long-chain ceramides, might impair permeability barrier function, exacerbating pathological changes (27, 28). TNF-α is primary inflammatory cytokine produced during the early cellular response to inflammatory stimuli which activate the cell towards the release of inflammatory cytokines (IL-6, IL-8, IL-12, IL-15, IL-17, IL-18) (29, 30). Chronic inflammation is also characterized by increased production of IL-12 and IL-8. Furthermore, both IL-8 and IL-12, are considered to be the markers of chronic inflammation and predominant Th1/Th17 and IL-17/IL-22 responses, under increased expression of IL-5 (31).

Cytokines-Th9 in AD

Increased serum IL-9 levels have been recently reported in AD patients (32). Furthermore, in addition to cytokines inducing allergen-specific IgE production, eosinophilia, and recruitment of inflammatory cells to inflamed tissues (33, 34), IL-9 could be crucial to the induction of pruritus. Although Th9 cells are characteristically pro-inflammatory, they also produce IL-10, an anti-inflammatory cytokine which down-regulates the synthesis of cytokines Th1 and/or Th2 cells during inflammatory tissue damage, favouring immune tolerance (35).

Cytokines-Th17 in AD

Th17 cells are a pro-inflammatory subset of memory CD4+ T cells involved in many autoimmune and allergic disorders (36). IL-17, also known as IL-17A, is a key cytokine in host defence infections, in the pathogenesis of autoimmune diseases (35, 38), as well as a critical factor in the pathophysiology of allergic diseases (36-38), inducing IL-1α, TGF-β, and IL-6 which, in turn, further promotes the generation of Th17 from naive T cells. Moreover, major serum IL-17 levels have been frequently detected in acute AD phase and rarely in the chronic phase of the disease (36-38). IL-17F (or IL-25), mainly produced by Th17 cells, is also released by

several other cell types (e.g., basophils, eosinophils, and DCs) (36-39). IL-25, influencing the production of pro-inflammatory cytokines, is a pivotal modulator of Th2-mediated diseases (39-46). Th17 cells also release IL21, a cytokine with pleiotropic effects on the innate and adaptive immune system. IL-21, in an autocrine manner, promotes Th17 cell differentiation (35), inhibits IgE production, and correlates inversely with IFN-γ production and the severity of AD (77). Finally, IL-23, also secreted by DCs in response to immune danger, strengthens IL-17A production, Th17 phenotype, neutrophil and eosinophil recruitment, and antigen-induced Th2 cytokine production (39-46).

Cytokines- Th22 in AD

Recent findings have discovered a new CD4(+) T-cell subset, known as Th22, by identifying a cell population dedicated to the synthesis of the IL-22, IL-13, and fibroblast growth factor (FGF) isoforms, but not involved in the release of IFN-y, IL-4, or IL-17 (47). However, during inflammatory tissue damage, Th22 could contribute to the amplification of the immune response by enhancing the TNF-α-induced cytokines and chemokines released by keratinocytes (47-51), supporting progressive activation of Th2/Th22 axis from acute to chronic phases (47-52).

CONCLUSIONS

AD, a chronic relapsing-remitting inflammatory skin condition mimicking numerous other diseases (53, 54), is also sustained by immunological imbalance. Although pathogenesis of AD is complex and still not fully understood, it is well known that cytokines, acting in an autocrine or paracrine manner, play a key role in the development, maintenance and flare-up of AD. Here, we summarize recent studies conducted on pathogenesis of AD, underlying the complexity of the inflammatory regulation. However, further studies are clearly needed to give deeper insight into the significant role of these cytokines in the pathogenesis of disease. In the light of this, chemokines, cytokines and their receptors could be considered as new promising therapeutic targets to reverse or to stop the development of AD and related comorbidities.

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ATOPIC DERMATITIS: IS THERE A ROLE FOR PROBIOTICS?

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that commonly presents during early childhood. In the last decades the prevalence of AD has increased, especially in western societies. This frequently relapsing inflammatory condition has a strong impact on the quality of life of patients and families. The recent advances in the understanding of this disease have paved the way for the development of new strategies for the prevention and treatment of AD. Among the new therapeutic options, there is increasing interest in the potential benefit of probiotic supplementation. It has been widely demonstrated that the human microbiota plays a fundamental role not only in the maintenance of intestinal homeostasis through the interaction between microorganisms and the innate immune system, but also in the microbiota-mediated development of adaptive immunity. In addition, several studies have demonstrated that probiotics are able to influence the composition of gut microbiota and may exert immunomodulatory effects. According to these promising results, the possible application of probiotics in the therapeutic management of allergic diseases has been investigated in many studies. In particular, a considerable body of literature has been published analyzing the effects of probiotics on patients with AD. In order to shed light on frequently conflicting results, we reviewed the data regarding the application of probiotics in AD, with the aim to provide a state-of-the-art assessment of the most important studies exploring the role of probiotics both in the prevention and treatment of AD.

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, that usually presents in early childhood (1). AD has been increasing in prevalence over time, with significant social and economic impact, and currently affects about 20% of children in developed countries (2). AD is a complex disease with a genetic predisposition strongly influenced by innate and adaptive immune responses, as well as environmental factors, including allergen exposure, irritants, microbes, diet, stress, and air quality (1,3). The fact that AD frequently affects more than one family member is consistent with the strong genetic background of this disease (4). Twin studies support a high rate of concordance; identical twins have a 7-fold increased risk for AD, and fraternal twins have a 3-fold increased risk (4). There are two primary disease models: first, AD as a skin disease, which primarily develops from intrinsic defects of epithelial cells and the skin barrier, resulting in numerous abnormalities of the innate and adaptive immunity ("outside-inside" hypothesis); second, that AD is primarily an immunologic disease with mechanisms related to overactivation of the immune system and Th2-dominated immune responses that impact on skin barrier function secondarily ("inside-outside" hypothesis). To date, It is likely that the combination of a continuous interplay between both hypotheses best explains the complexity of AD (1). A mutation in the filaggrin (FLG) gene, responsible for an important component of the skin barrier, can be found in up to 10% of people of European ancestry (5); patients with FLG mutations have been found to have dry skin and early-onset AD that is more persistent and often associated with asthma, food

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allergy, and microbial infection (6). Aside from FLG, AD has been associated with variants in other genes involved in skin barrier dysfunction and also in innate and adaptive immune responses control (1,7).

It is noteworthy that AD skin lesions are driven by underlying immune pathways (8). The acute initiation of AD skin lesions is associated with Th_2, Th_{22}, and also Th_{17} cytokine activation; likewise, in patients with chronic AD, intensification of Th_2 and Th_{22} activation occurs with the appearance of a significant but non complete switch to Th_1, with an increase in IFN-γ levels that contributes to the inflammatory response and causes keratinocyte apoptosis (1,9). AD disease severity has also been linked with other minor immune mediators dysfunction (10, 11, 12).

Patients with established AD have a combination of skin barrier dysfunction and skin inflammation driving their skin disease. Therefore keys to the successful management of AD should include skin hydration and skin barrier repair, topical antiinflammatory medications (topical corticosteroids or calcineurin inhibitors), control of infection, and elimination of factors (including allergens, irritants, and emotional triggers) that might exacerbate the scratch-itch cycle. Treatment should use a stepwise approach that is dependent on the severity of skin disease (1, 13, 14).

The recent advances in the understanding of this disease have paved the way for the development of new strategies for the prevention and treatment of AD. Among the new therapeutic options, there is increasing interest in the potential benefit of probiotic supplementation.

It has been widely demonstrated that the human microbiota plays a fundamental role not only in the maintenance of intestinal homeostasis through the interaction between microorganisms and the innate immune system, but also in the microbiota-mediated development of adaptive immunity (15). Providing a unique interaction with the host immune system, the gut flora might protect from developing early allergic symptoms (15). Probiotics are "live microorganisms that, when administered in adequate amounts, may confer a health benefit on the host" (16). They have been widely reported to alleviate lactose intolerance, suppress diarrhea, reduce irritable bowel symptoms, and prevent inflammatory bowel disease. The probiotics most commonly used are *Lactobacilli*, *Bifidobacterium*, and *Enteroccoci*, also part of the intestinal microflora (17). Several studies have demonstrated that probiotics are able to influence the composition of gut microbiota and may exert immunomodulatory effects (18, 19, 20, 21). According to these positive results, the possible application of probiotics in the therapeutic management of allergic diseases has been recently investigated. In particular, a considerable body of literature has been published analyzing the effects of probiotics on patients with AD. In order to shed light on frequently conflicting results, we reviewed the data regarding the application of probiotics in AD, with the aim to provide a state-of-the-art assessment of the most important studies exploring the role of probiotics both in the prevention and treatment of AD.

MATERIALS AND METHODS

We searched the electronic database, PUBMED using the free text words: *probiotics*, *Bifidobacterium*, *Lactobacillus*, combined with the terms: *children* and *atopic dermatitis* and *atopic eczema*. Our search was limited to studies available in English and published between 2008 and October 2014. We included in our analysis three studies published before 2008 because they are considered pivotal researches in this field. We also searched the Cochrane Central Register of Controlled Trials (CENTRAL) in order to summarize the most important studies published before the last five years.

RESULTS

Probiotics in the Prevention of AD

Double-blind, randomized, placebo-controlled clinical trials have been published in order to investigate the efficacy of probiotics in preventing AD.

One of the first studies evaluating the role of probiotics in the prevention of atopic diseases is a trial conducted by Kalliomäki et al. *Lactobacillus rhamnosus* strain *GG* (ATCC 53103*)* was administered prenatally to mothers who had at

least one first-degree relative or partner with atopic eczema, allergic rhinitis, or asthma, and postnatally for six months to their infants. The frequency of atopic eczema for the infants in the probiotic group was half that of the placebo group (15/64 (23%) vs 31/68 (46%); relative risk 0·51 (95% Cl 0·32—0·84)). The number needed to treat was 4·5 (95% Cl 2·6—15·6). (22). This study showed that perinatal administration of the probiotic *Lactobacillus GG* reduces incidence of atopic eczema in at-risk children during the first 2 years of life.

These findings were confirmed with significantly decreased cumulative risks of developing eczema in children aged four and seven. In particular, at 4 years of age, 14 of 53 children receiving *Lactobacillus* had developed atopic eczema, compared with 25 of 54 receiving placebo (relative risk 0.57, 95% CI 0.33-0.97) (23). Moreover, in order to evaluate the cumulative effect of the probiotic intervention during the first 7 years of life, the same researchers re-examined the study cohort at the age of 7 years. The follow-up was completed by 116 of 159 (73%) children, 62 of 82 (76%) in the placebo group and 53 of 77 (69%) in the *Lactobacillus GG* group. The cumulative risk for developing eczema during the first 7 years of life was significantly lower in the *Lactobacillus GG* group than in the placebo group (42.6% vs 66.1%; RR, 0.64; 95% CI, 0.45-0.92). The risk of eczema was significantly reduced in the *Lactobacillus GG* group compared with the placebo group (odds ratio, 0.58; 95% CI, 0.35-0.94; *P* = .027) (24). These results suggest that the preventive effect of *Lactobacillus GG* on atopic eczema extends beyond infancy.

These three studies by Kalliomäki et al. paved the way for more researches in this field.

In order to summarize the heterogeneous results of trials on this topic, the Cochrane Collaboration evaluated the preventive role of probiotics in atopic diseases through a exhaustive meta-analysis that included searches of the Cochrane Central Register of Controlled Trials, MEDLINE (1966 - February 2007), EMBASE, PREMEDLINE, abstracts of conference proceedings and citations of published articles, and expert informants. Twelve studies resulted eligible for inclusion. The authors concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease. It is important to highlight that a subgroup analysis of five studies reporting the outcomes of 1477 infants found a significant reduction in infant eczema (typical RR 0.82, 95% CI 0.70, 0.95). However, this effect was not consistent between studies and, when the analysis was restricted to studies reporting atopic eczema (confirmed by skin prick test or specific IgE), the findings were no longer significant (typical RR 0.80, 95% CI 0.62, 1.02) (25).

After the above cited meta-analysis further clinical trials have been published in order to clarify the conflicting results.

In a study by Wickens et al, pregnant women were randomized to take *Lactobacillus rhamnosus* HN001, *Bifidobacterium animalis* subsp *lactis* strain HN019 or placebo daily from 35 weeks gestation until 6 months if breast-feeding, and their infants were randomized to receive the same treatment from birth to 2 years (n = 474). The infant's cumulative prevalence of eczema and point prevalence of atopy, using skin prick tests to common allergens, was assessed at 2 years. It resulted that supplementation with *Lactobacillus rhamnosus*, but not *Bifidobacterium animalis* subsp *lactis*, substantially reduced the cumulative prevalence of eczema, but not atopy, by 2 years. In particular, infants receiving *Lactobacillus rhamnosus* had a significantly reduced risk (P = .01) of eczema (hazard ratio (HR), 0.51; 95% CI, 0.30-0.85) compared with placebo (26).

In two subsequent trials the same study group investigated the associations of supplementation with *Lactobacillus rhamnosus* HN001 or *Bifidobacterium animalis* subsp *lactis* strain HN019 with allergic disease and atopic sensitization among these children at age 4 years (2 years after stopping probiotic supplementation) and at age 6 years. The cumulative prevalence of eczema by 4 years (Hazard ratio (HR) 0.57 (95% CI 0.39-0.83)) was significantly reduced in the children taking *Lactobacillus rhamnosus*, while there were also non significant reductions in the cumulative prevalence of SCORing Atopic Dermatitis (SCORAD) score ≥ 10 (HR 0.74 (95% CI 0.52-1.05) (27). Moreover, considering the children

at age 6 years, supplementation with *Lactobacillus rhamnosus* was associated with significantly lower cumulative prevalence of eczema (HR = 0.56, 95% CI 0.39-0.80), SCORAD ≥ 10 (HR = 0.69, 0.49-0.98) and SPT sensitization (HR = 0.69, 95% CI 0.48-0.99). The point prevalence of eczema (RR = 0.66, 95% CI 0.44-1.00), SCORAD ≥ 10 (RR = 0.62, 95% CI 0.38-1.01) and SPT sensitization (RR = 0.72, 95% CI 0.53-1.00) were also reduced among children taking *L. rhamnosus*. However, supplementation with *Bifidobacterium animalis* subsp *lactis* did not affect the prevalence of any outcome at age 4 or 6 years (28).

Thanks to these three trials by Wickens et al. and other well-conducted studies, it soon became apparent that not all probiotics have the same efficacy. It is now well established that probiotic effects depend on the specific strain, therefore not all probiotic strains might be good candidates for AD prevention (29). Moreover, the heterogeneity of the results could be partially explained by the heterogeneity of the effect of different strains. Only a limited number of specific strains, and in particular *Lactobacillus rhamnosus* (30, 31), have been extensively studied with promising results. However, to date, the ideal combination and concentration of microorganisms, if existing, remains to be found.

Another important aspect that could explain the variability of the results is the difference in the study design of trials. In particular, most of the studies differ with respect to the time of probiotic use (pregnancy or early life) or the subject receiving probiotics (mother, child, or both).

A pivotal study by Rautava et al. investigated whether maternal probiotic supplementation during pregnancy and breastfeeding reduces the risk of developing eczema in high-risk infants. Mothers with allergic disease and atopic sensitization were randomly assigned to receive *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 (LPR+BL999), *Lactobacillus paracasei* ST11 and *Bifidobacterium longum* BL999 (ST11+BL999), or placebo, beginning 2 months before delivery and during the first 2 months of breastfeeding. 205 infants completed the follow-up until the age of 24 months. The risk of developing eczema during the first 24 months of life was significantly reduced in infants of mothers receiving LPR+BL999 (odds ratio (OR), 0.17; 95% CI, 0.08-0.35; P < .001) and ST11+BL999 (OR, 0.16; 95% CI, 0.08-0.35; P < .001). However, the risk of atopic sensitization in the infants was not influenced by probiotics (32).

A positive effect of the supplementation with probiotics is reported also in a recent study involving a large population-based pregnancy cohort of 40614 children. Consumption of probiotic milk in pregnancy was associated with a slightly reduced relative risk (RR) of atopic eczema at 6 months (adjusted RR, 0.94; 95% CI, 0.89-0.99) compared with no consumption during pregnancy (33).

In a recently published meta-analysis of 16 studies by Panduru et al., the supplementation with probiotics confers protection against atopic dermatitis occurrence (OR = 0.56, P = 0.0001). Moreover, a subgroup analysis showed that the prenatal administration of probiotic followed by postnatal administration was protective (OR = 0.54, P = 0.001) unlike the only administration in postnatal period (OR = 0.89, P = 0.59) (34).

Despite these positive results, it is evident that, even after the analysis of several well-conducted studies, the precise role of probiotics in prevention of atopic dermatitis is still not clear.

Meta-analytic evidence suggests probiotics may be effective for preventing eczema, but the heterogeneity of the probiotic products considered and the difference in the study protocols and outcomes limit the possibility of an unambiguous interpretation of the results. As stated in the World Allergy Organization position paper on the Clinical Use of Probiotics in Pediatric Allergy (35), it is not possible to issue clear recommendations at present.

Probiotics in the Treatment of AD

Although there are promising results regarding the preventive role of probiotics in the development of atopic dermatitis, there are less convincing data about their effects as treatment of the established disease.

The results of a meta-analysis by Lee et al. reinforced this concept showing a potential preventive role of probiotics in pediatric atopic dermatitis, but no clinically significant changes in the severity of the

disease with the treatment of probiotics (36). In the meta-analysis by Michail et al., only a modest effect of probiotics in reducing the SCORAD score (mean change from baseline, -3.01; 95% confidence interval, -5.36 to -0.66; P = .01) was shown (37).

As for the investigations on the preventive efficacy of probiotics, in order to summarize the heterogeneous results of trials on this topic, the Cochrane Collaboration evaluated the therapeutic role of probiotics in eczema through a exhaustive meta-analysis. The authors analyzed twelve randomized controlled trials involving 781 participants and concluded that the results showed that probiotics are not an effective treatment for eczema (38).

However, it is important to highlight that the clinical trials investigating the treatment with probiotics are smaller and more heterogeneous than their preventive counterparts, so they are less likely to show significance. Moreover, recently published study demonstrated more positive results. Supplementation with probiotic *Lactobacillus plantarum* CJLP133 in children aged 12 months to 13 years resulted in a greater reduction in the SCORAD score at week 14 in the probiotic group than in the placebo group ($p = 0.044$). The mean change in the SCORAD score from weeks 2 to 14 was 9.1 in the probiotic group, which was greater than the mean change of 1.8 in the placebo group ($p = 0.004$) (39). Drago et al. found that *Lactobacillus salivarius* LS01 reduced SCORAD scores of 38 patients with moderate to severe AD (40). In a clinical trial by Iemoli et al., 48 patients with atopic eczema were randomized to receive a probiotic combination (*Lactobacillus salivarius* LS01 and *Bifidobacterium breve* BR03) or placebo for 12 weeks; patients receiving probiotics showed a significant improvement in clinical parameters (SCORAD, $P<0.0001$), while none of these changes were observed in the placebo group (41).

Although these studies are promising, the small number of subjects prevents generalization of the results.

Considering the evidence analyzed, although a promising role has been found in individual trials, meta-analyses have not shown efficacy in the treatment of atopic dermatitis. These conflicting results could possibly be explained by some considerations.

As noted regarding the preventive role of probiotics, there are considerable differences in the probiotic strains and dosages that were used in the treatment studies. Moreover, the number, age and eczema severity of the participants and the treatment period differ between studies.

CONCLUSIONS

We analyzed the most recent trials evaluating the possible role of probiotics for prevention and treatment of atopic dermatitis.

Evidence from trials and meta-analysis suggest that probiotics may be effective for preventing atopic dermatitis while there is currently less evidence that probiotics are clinically effective for treating established eczema.

It is important to highlight that the efficacy of probiotic interventions is strongly dependent on the specific strain used. It is known that each probiotic strain is unique with specific local and systemic immunomodulatory effects and influences the gut microbiota in different ways. The effects of probiotic combinations may differ from individual probiotics and their efficacy could also be directly affected by production methods and delivery food matrices. Furthermore, the probiotic effect is obviously related to the endogenous gut microbiota, host immune system, clinical condition and diet.

The heterogeneity of the probiotic products considered and the difference in the study protocols and outcomes represent a limitation for evidence-based recommendations in the generalized use of probiotics.

Further studies are needed to standardize study designs, bacterial strains, dosages, and durations in order to clarify the possible application of probiotics in the prevention and treatment of atopic dermatitis.

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CHILDHOOD IDIOPATHIC NEPHROTIC SYNDROME AND ATOPIC DISEASES: IS THERE A RELATIONSHIP?

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Nephrotic syndrome is a condition of massive proteinuria that leads to hypoalbuminaemia and oedema. In the pediatric age, the most common form of nephrotic syndrome is childhood idiopathic nephrotic syndrome (CINS). Although the etiological mechanisms underlying CINS are still unclear, the disease is considered to be immune-mediated. Several studies have previously reported a possible association between CINS and atopy, with the latter defined as abnormal immunoglobulin-E response on the background of a T-helper 2 (Th2)-driven immune system. In fact, both experimental and clinical studies have suggested that idiopathic nephrotic syndrome can be associated and/or triggered by a wide array of atopic diseases, though this remains a highly controversial topic. Exposure to inhalant-allergens (and/or introduction of food-allergens) has been previously correlated with the onset and/or the relapse of CINS in some children and a significant worse response to steroid therapy has been also described in reports of CINS associated to concomitant atopic diseases. In this review, we analyzed previous studies with the aim to clarify, basing on the existent literature, the association between atopy and idiopathic nephrotic syndrome. Additionally, we also speculated on the underlying immunological pathways that could potentially make some children prone to both CINS and atopic diseases.

Nephrotic syndrome (NS) is a condition of massive proteinuria leading to hypoalbuminaemia and oedema (1). In the pediatric age group, the most common form of NS is childhood idiopathic nephrotic syndrome (CINS), with an incidence of 2-7 cases per 100,000 children and a prevalence of nearly 16 cases per 100,000 (2). There are different etiological and/or histological-defined causes of CINS, including minimal change disease (MCD) and, less commonly, focal segmental glomerulosclerosis (FSGS). Although the exact pathophysiological mechanisms of CINS are largely unknown, the condition is believed to be immune-mediated in most cases (3). Experimental studies showed that circulating *permeability factors* of T-cell origin (3) seem to impair the permeability of the glomerular podocytes, allowing the passage of albumin and/or other negatively charged proteins, subsequently leading to proteinuria and interstitial oedema (4); additionally, the oedema (with intravascular hypovolemia) is often worsened by endocrine factors (i.e., activation of the renin aldosterone system) which are likely responsible for other CINS-related complications (5). Notably, it has been speculated that

Key words: childhood idiopathic nephrotic syndrome; atopic sensitization; allergic diseases; children.

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CINS is a podocyte disorder mediated by a "2-hit" pathophysiological mechanism (6, 7). Initially, there is a release of several pro-inflammatory interleukins (IL) (e.g., IL-4, IL-5, and IL-13) in immune cell and podocytes, activated by different external stimuli (e.g., infectious pathogens, microbial products, food and/or inhalant allergens) (8). At a later time, B-cell IgE isotype switching, modulation of inflammation and the further recruitment of T-cells, likely lead to the secondary (i.e., anatomical and/or functional) glomerular changes typical of MCD or FSGS (7, 8). Some previous studies proposed a possible link between CINS and atopy, characterised in a hereditary predisposition to produce an exaggerated T-helper (Th2)-driven and (allergen-specific) immunoglobulin (Ig)-E mediated immunological response (6). The aim of this review is to summarize the current knowledge on the relationship between atopy and CINS.

Immune system as a link between atopy and CINS

In 1974, Shalhoub hypothesized that the proteinuria in CINS was due to a circulating factor released by T cells (3). His speculations were based on different observations: (i) CINS is usually triggered by viral infections (e.g. respiratory tract infections), which lead to T cell-mediated immunity; (ii) CINS may frequently occur concomitantly with T-cell disorders (e.g., Hodgkin's disease); (iii) CINS responds to therapeutic agents generally used to suppress T cell-mediated immunity (e.g., steroids and cyclophosphamide); lastly, (iv) CINS show an absence of humoral (e.g., immunoglobulin and complement) components in glomeruli. Recently, a pathophysiological role of the podocyte in the onset of CINS was also recognised (9, 7). It is known that podocytes express (spontaneously or after stimulation) various interleukins (e.g., IL-4, IL-13) and CD80, a T-cell stimulatory factor that is also involved in the actin rearrangement and in the organization of podocytes pedicels. All this results in impaired glomerular permeability, leading to the onset of proteinuria (10). Additionally, urinary CD80 levels were found to be elevated in patients with onset and/or relapse of CINS and lowered during remission phase of CINS (9). Interestingly, the critical role of the IL-4/IL-13/CD80 axis was also studied in other conditions, including atopic diseases (7, 12). Atopy is a T-helper 2 (Th2)-driven and IgE-mediated chronic inflammation of a target organ (8, 13-19). Activated Th2 cells release IL-4, IL-5, and IL-13 (6, 8). IL-4 and IL-13 are critical in B-cell IgE isotype switching (6, 8), and IL-13 is also involved in the modulation of inflammation and the recruitment of monocytes and T-cells (6, 8). Notably, experimental studies showed that IL-13 may act at the glomerular level, down regulating nephrin, podocin and dystroglycan and up regulating CD80, likely due to a podocyte injury (20). Additionally, the induction of CD80 in podocytes has been found to disrupt the filtration barrier, resulting in spontaneous proteinuria (21, 22). Lastly, these data might justify the "allergic diathesis" noticed in some CINS patients, and further confirm the relationship link between atopic and non-atopic diseases (6, 7).

CINS, atopic sensitization, and allergic diseases

It is still unclear whether atopic status in some children with CINS is merely coincidental or represents an underlying etiological and/or contributing factor in the development of the pathological proteinuria (6). Some authors previously reported a predictive positive value of serum IgE levels to assess disease activity in patients with CINS: decreased serum IgE levels have been measured during the remission of CINS and increased IgE values have been detected during the relapse (23, 24). On the basis of these results, the authors suggested that serum IgE levels could be potentially utilised as prognostic factor in some children with CINS (24). Additionally, in these studies both the children with steroid-sensitive (SS) and with steroid-resistant (SR) CINS had serum IgE levels significantly higher (compared to normal controls) prior the steroid treatment and those with SR-CINS were found to have the higher serum IgE levels after the treatment, compared to the SS-CINS group (23, 24). These findings suggested that response to steroids in CINS may possibly correlate with a decreased Th2-immune activity and SS patients, showing an excessive Th1 response, reported a minor expression of Th2 cellular subset (25). Of note, several additional and/or external factors may bias the levels of serum IgE (26). In fact, most of the children affected by CINS underwent corticosteroid therapy. Steroid treatments may deregulate several

homeostatic processes in various organs and/or tissues, also leading to an impairment (th2-driven) of immune response and of other homeostatic processes (26, 27). Moreover, some studies of CINS reported only serum total IgE levels increased, while specific IgE antibodies were not found (6). Therefore, the increase of serum IgE levels during the acute or relapse phases of CINS may likely reflect an underlying process of dysregulation of the humoral immune response, rather than a direct involvement of IgE in CINS pathophysiology (28). Notably, the findings that the onset of CINS can occur in association with atopic diseases led some authors to consider allergens as triggering factors of CINS. The potential association between CINS and atopic diseases was also suggested by case-studies and case-series that described children in which CINS seemed to be triggered by specific allergens (12, 22, 29). In these reports, the exposure to inhalant (e.g., grass pollens, rag-weed pollens, fungi, and house dusts) and/or the introduction of food allergens (e.g., cow's milk, fish, chicken, ovalbumin) were associated to the onset and/or the relapse of CINS. However, other authors suggested caution in evaluating the role of allergens as trigger factors of CINS (6, 29, 30) and critically considered the above reported associations due to the fact that most allergens were only identified by using limited methods (e.g., radioallergosorbent test) and, additionally, in the above reported associations a true "challenge" to induce CINS, by exposing to the suspected causative allergen, was not recorded. Notably, there was also no evidence for seasonal relapse of CINS as in the studies where the disease was associated to allergens exposure (12, 29), the initial episodes of CINS were distributed indistinctly throughout the year. However, allergens have been putatively implicated in triggering CINS but there is no evidence from previously published studies that blocking the specific inhalant- and/or food allergen may prevent relapse of proteinuria or ameliorate the clinical course of CINS. This further corroborates the suggestion that atopic response is merely the result of the immune deregulation observed in CINS patients.

CONCLUSIONS

CINS and atopic diseases are both frequent disorders in the paediatric age group. Although several theories have been proposed to date to explain the underlying pathophysiology of CINS, the exact underlying pathophysiological mechanism still remains unclear. Many previous studies focused on the relationship between CINS and atopy in different paediatric populations (31). Later studies suggested an increased incidence of atopic diseases in children with CINS (32), and significantly raised IgE levels in the context of nephrotic syndrome were also reported by other studies (33). All of these findings led some authors to speculate a potential role of atopy in the pathophysiology of CINS and to consider some allergens as triggering factors in the onset (and/or relapse) of proteinuria (34). Although some experimental studies highlighted a possible relationship between atopy and CINS (20), most of the clinical studies published to date in this regard showed a marked variability in terms of serum IgE levels and incidence of atopic diseases and/or atopic sensitization in children with CINS (23, 24). Further studies have to be carried out in future to definitively assess the exact role of atopy in the natural history of CINS and to provide additional insights on the effects of atopic response in the physiology and function of podocytes.

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CYSTIC FIBROSIS AND ANTIBIOTIC HYPERSENSITIVITY: PRESENT KNOWLEDGE AND PRACTICAL APPROACH

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Cystic fibrosis is one of the most common fatal genetic diseases (1 in 2500 births). The defect causing the disease is localized on the 7q31 gene, which codifies for the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) transmembrane protein. CFTR is a chloride channel localized on the epithelial cells of the mucosa of the respiratory tract, pancreatic ducts, biliary tree, intestine, vas deferens, and sweat glands. More than 2000 different mutations are currently known; some are prominent or relatively frequent, ranging from one population to another. The most frequent complications of cystic fibrosis are those affecting the bronchial tree. Patients suffer from recurrent lung infections, which involve a progressive loss of lung function. The pulmonary infections are frequent or chronic and limit the quality of life of patients. In addition to being enormously exposed to antibiotics, they have many more opportunities to develop hypersensitivity reactions to these molecules. Only a complete allergy work-up with a detailed analysis of the clinical history, skin tests and provocation test can show if the patient has actually experienced an allergic hypersensitivity reaction. Desensitization is to be considered as a treatment that may help patients benefit from antibiotic treatment in those cases in which they have a proven allergy to a certain molecule.

Cystic fibrosis today, lung disease, and need for antibiotics

Cystic fibrosis (CF) may be suspected prenatally, through the analysis of the intestinal echogenicity in fetal ultrasound. At birth, 10-20% of CF patients suffer from meconium ileus, which may be complicated by peritonitis, volvulus or ileal atresia (1). A neonatal screening for cystic fibrosis, currently available in several Countries, is based on the determination of immunoreactive trypsin levels in peripheral blood. If the test is positive (0.5 to 1% of births), patients should be investigated for genetic mutations. After the first 6 months of life, assaying trypsin may give a lot of false negatives. Therefore, after this age, it is preferred to investigate patients presenting with typical CF clinical signs, through a sweat test: an increase in chlorine above 60 mEq/l is considered as pathological. Patients reporting two positive sweat tests need a complete genetic evaluation to characterize the responsible mutations. Nevertheless, some patients present with mild symptoms and may be diagnosed later.

Typical signs of the disease in its classic form include oily, fetid diarrhea associated with steatorrhea, rectal prolapse, failure to thrive, exocrine pancreas failure, diabetes, recurrent bronchitis, pneumonia, chronic rhinosinusitis, nasal polyposis, and, rarely, hepatobiliary disease, with obstructive

Key words: allergy work-up, antibiotics, cystic fibrosis, desensitization, drug allergy

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jaundice and cirrhosis (1). The evolution of the disease is very variable, but today, even patients with the classic form of CF and homozygous mutations (including the most common ΔF508 mutation) have a life expectancy greater than 30 years.

The prognosis is now strictly linked to the respiratory disease, whose current treatment has a major impact on patients' quality of life and overall survival (1). However, most of the patients still die because of respiratory failure. Moreover, lung transplantation does not eliminate the recurrence of lung infections, but allows an increase in life expectancy. The main treatments that may postpone transplantation include aerosol administration of rhDNase, chest physiotherapy associated with bronchial drainage and physical activity. Certain antibiotics such as azithromycin may be administered chronically, at anti-inflammatory doses, while others, such as inhaled tobramycin or colimycine, help fight against chronic lung infections. However, in the presence of pulmonary exacerbations, patients need high doses of antibiotics.

Interestingly, some European CF centers recommend the regular administration of intravenous antibiotics therapy, regardless of the clinical status of the patient (1). Different bacteria infect the lungs of CF patients throughout their life. During the first year, enteric organisms are mainly isolated. Over time, patients may acquire one or more pathogenic bacteria like Staphylococcus aureus, Haemophilus influenzae and Pseudomonas aeruginosa in their airways. About 60% of children aged 6 to 10 years are colonized by S. aureus and almost 80% of those aged 25-34 years are colonized with P. aeruginosa (1). Some clinical studies have suggested that nearly 80% of patients at the age of 10 years are colonized with P. aeruginosa, suggesting an even earlier acquisition of the infection. In advanced stages of the disease, patients may acquire Burkholderia cepacia infections in their airways (1).

Several classes of antimicrobial drugs may be selected during a pulmonary exacerbation. The goal of antibiotic therapy in CF patients is to remove the airways bacterial infection even though most of the times it simply aims at reducing the severity of the exacerbation, in chronically colonized patients. Exceptions to this approach may include aggressive antimicrobial therapy to eradicate the first cultures of P. aeruginosa in young children and multidrug therapies centered on the elimination of atypical organisms such as B. cepacia and Non-Tubercular Mycobacteria. Most commonly administered antibiotics include penicillins, aminoglycosides, and quinolones.

Hypersensitivity to antibiotics and cystic fibrosis

Allergic reactions to antibiotics are the expression of immune hypersensitivity reactions. It is always important to identify in the clinical history when the reaction occurred, in order to classify it as immediate, semi-retarded or delayed, and to better understand the immunological mechanisms involved (2-3). As for immediate reactions, clinical manifestations appear in less than one hour after drug administration. In semi-delayed reactions, the interval between drug administration and the reaction is between 1 and 72 hours. Delayed reactions are those appearing more than 72 hours after the drug intake.

The incidence of allergic hypersensitivity drug reactions in the general population is well known for β-lactams, and it affects 1-10% of the population (4). In 1970 to penicillin allergy was first described in CF patients, but since then many studies have been conducted (4-11). Even though many of them are questionable as to their methods, it seems that the prevalence of allergic hypersensitivity to β-lactams in CF patients is higher than in the general population, affecting from 6 to 22.5% of patients. Among the β-lactams, ureidopenicillins (mezlocillin and piperacillin) and carbapenems (imipenem) are more associated with allergic reactions (25% and 24.6% respectively) with respect to cephalosporins (6.5%) and carbossipenicillines (1.6%) (8). A 2012 study has confirmed that drug allergy to β-lactams in CF patients is clinically important, but it also pointed out that many patients complaining of a history suggestive of drug allergy, are actually not allergic. (12) The Authors highlighted the need to perform a complete allergy work-up in CF patients, since considering them as allergic on the basis of their clinical history alone may result in a loss of treatment opportunities, and of life expectancy (12).

Clinical manifestations reported by patients include, in the order of frequency, hyperthermia, which may arise from the seventh day of therapy and disappears 48 hours after the last dose; cutaneous manifestations, mainly maculopapular rash, hives, facial swelling, or itching; and anaphylactic shock (8-11). Unfortunately, knowing the exact timing and the characteristics of a reaction does not necessarily provide complete information about the allergic mechanism causing the reaction. Indeed, many delayed reactions have similar characteristics to immediate reactions, and sometimes it is difficult to distinguish between IgE and not IgE-mediated reactions (13).

As for aminoglycosides, the incidence of allergic reactions is high for neomycin and streptomycin (greater than 2%), while occasional for gentamicin and amikacin (0.1 and 2%), and rare for kanamycin (between 0.1 and 0.5%). Possible cross-reactions between aminoglycosides may not be ruled out. Patients usually experience generalized reactions (especially fever with streptomycin, while anaphylactic shock is rare), and less frequently cutaneous signs only (urticaria, maculopapular rash and contact dermatitis, especially with streptomycin). Sulfites, contained in many aminoglycosides, may be responsible for several hypersensitivity reactions to aminogycosides (14).

Quinolones are rarely associated with allergic hypersensitivity reactions. Patients may experience systemic reactions (anaphylaxis, laryngeal edema), or isolated cutaneous signs (pruritus, bullous eruptions), or hematological disorders (eosinophilia, leukopenia, hemolytic anemia) (15-17). Hypersensitivity reactions may occur with all the different generations of quinolones (16,18).

Vancomycin is associated to the same type of reactions described for quinolones, with a prevalence of skin reactions. Allergic hypersensitivity to Vancomycin typically occurs in patients suffering from endocarditis and β-lactam allergy (14). The most common and most impressive side effect of vancomycin is the red man syndrome: this reaction is characterized by pruritus, erythematous

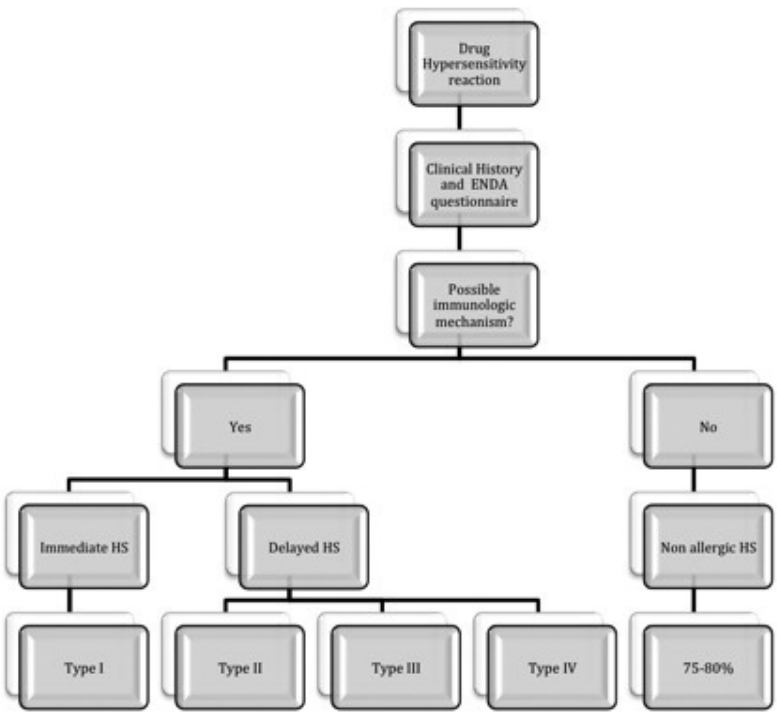

Fig. 1. *Allergy assessment in Cystic Fibrosis patients.ENDA: European Network for Drug Allergy; HS: Hypersensitivity*

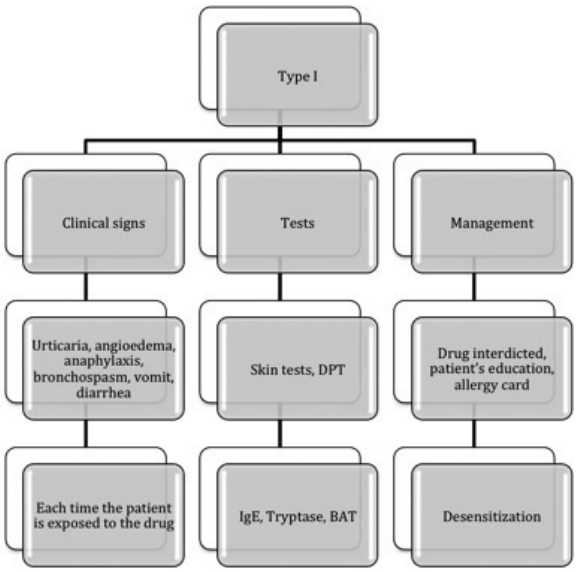

Fig. 2. *Management of Cystic Fibrosis patients with proven Type I Hypersensitivity. DPT: Drug Provocation Test; BAT: Basophil Activation Test*

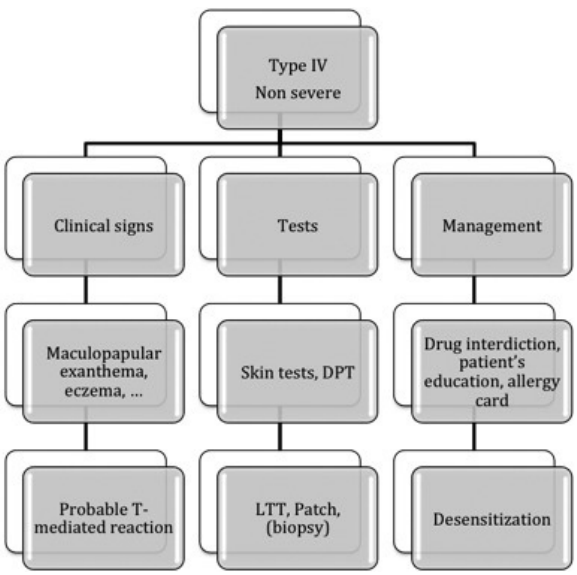

Fig. 3. *Management of Cystic Fibrosis patients with proven non-severe Type IV Hypersensitivity DPT: Drug Provocation Test; LTT: Lymphocyte Transformation Test*

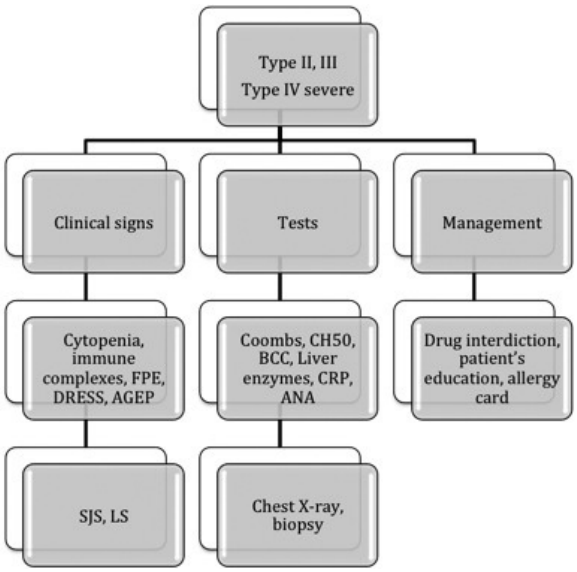

Fig. 4. *Management of Cystic Fibrosis patients with Type II, or Type III, or proven severe Type IV Hypersensitivity*
FPE: Fixed Pigmented Erythema; DRESS: Drug Reaction with Eosinophilia and Systemic Symptoms; AGEP: Acute Exanthematous Generalized Pustulosis; SJS: Stevens-Johnson Syndrome; LS: Lyell Syndrome; BCC: Blood Cells Count

rash on the face, neck and trunk, accompanied by laryngeal edema to shock in the most severe cases. However, this syndrome is related to a non-specific histamine release by direct activation of mast cells and basophils, and is not immunologically mediated. Therefore it is not to be considered as an allergic form of hypersensitivity.

Allergy approach in CF patients

Antibiotic allergy is a major problem for CF patients. The work-up begins by collecting a detailed clinical history, to assess the timing of the reaction and its severity and therefore to assess the best tests to perform. The European Academy of Allergy and Clinical Immunology (EAACI) convened a multidisciplinary working group to standardize the diagnosis of drug allergy (ENDA: European Network for Drug Allergies). The group proposed a questionnaire (19), general recommendations on the practice of skin testing, and recommendations for provocation tests (20). What seems important is that in front of each patient, the clinical history is fundamental to decide the best approach to identify and/or exclude a possible allergic form of hypersensitivity (Figure 1).

For immediate reactions (anaphylactic shock, bronchospasm, laryngeal edema, urticaria, maculopapular rash), immediate skin tests should be performed, including skin prick tests (SPT) and intradermal tests (IDR). These tests must be conducted to clarify the guilt of the drug and to search for a possible cross-reactivity between antibiotics in the same class (Figure 2). In case of delayed reaction, skin tests include delayed IDR and/or patch tests (Figure 3). Severe reactions like Stevens-Johnson syndrome, Lyell's syndrome, or type II and type III allergic reactions, according to the Gell and Coombs classification, should not be investigated, if the drug is likely responsible for the reaction. In these cases, the drug has to be considered as contraindicated for

the patient (Figure 4) (21-24).

SPT are used to explore immediate hypersensitivity reaction (25). Preferably they should be performed 4 to 6 weeks after the supposed allergic manifestation. For safety purposes, SPT should be the first test included in the allergic work-up, followed, if negative, by IDR. It is recommended to verify IDR 24–72 hours or even a week after the test is performed, when the clinical manifestations suggest a delayed reaction, or whenever patients report a history, which does not sufficiently discriminate between an immediate and a non-immediate reaction. Immediate skin tests are able to demonstrate the IgE-dependent mechanism related to the hapten reactive drug. Their sensitivity and predictive value varies depending on the involved drugs. They are high for some molecules (penicillins, cephalosporins, pristinamycin), but low or unknown for others (quinolones, macrolides). As for beta-lactams, these tests are standardized, and increasing concentrations of 0.25, 2.5 and 25 mg/ml should be assessed. In case of severe reactions, such as anaphylactic shock, it is prudent to begin IDR at lower concentrations. For other antibiotics, there are few standardized protocols. Patch tests are of great value for the diagnosis of drug contact dermatitis (especially for streptomycin, penicillin). It seems that the association between a positive delayed IDR and a positive patch test for the same drug is of great diagnostic value in delayed skin reactions (26-28).

A positive skin test highlights the immunological mechanism of the previous hypersensitivity reaction to the drug. A negative test does not imply that the patient is not allergic. In these cases, a drug provocation test (DPT) is needed to complete the work-up.

DPT must be conducted in a specialized hospital and after obtaining a signed informed consent from the patient. Its purpose is primarily to exclude a form of allergic hypersensitivity to an antibiotic. Drug administration begins at very low doses, which increase gradually step by step, in the absence of an allergic reaction, according to different protocols. The cumulative dose should be close to the final daily dose of the drug. If an allergic reaction occurs, the test should be stopped and the patient should be considered as allergic to the molecule, and an alternative drug should be find (Figure 2 and Figure 3). DPT should not be performed if the drug is rarely prescribed and/or if there are alternative drug that may be used, and when the reported reaction is severe (bullous forms, DRESS, erythematous pustulosis, acute vasculitis) (Figure 4). DPT is the test of maximum sensitivity, but it has to be performed under close supervision and in specialized centers only, where an Intensive Care Unit is available, in case of possible severe reactions to the test itself (29).

Therapeutic possibilities

When a CF patient has an allergic hypersensitivity to an antibiotic, different treatment options should be considered. First of all, it is mandatory to reassess the absolute necessity of the molecule. Unfortunately, there are few treatment options available in the population of patients with cystic fibrosis when organisms such as Pseudomonas aeruginosa and B. cepacia complex are isolated (13). Moreover, it has to be underlined that, with time, CF patients become resistant to most of the available antibiotics, if not to all kinds of antibiotics. Therapeutic options are therefore chosen on the basis of which molecule usually works the best on the pathogen, administered at very high doses. Two antibiotics that are often administered to CF patients may be associated with allergic hypersensitivity reactions, such as tobramycin and aztreonam. In these patients, the use of aerosol tobramycin and aztreonam lysine appears to be a viable alternative (30,31). Nevertheless, these antibiotics are prescribed as long-course therapies to prevent respiratory exacerbations in patients with chronic lung infections. Therefore, even though some authors propose an inhaled approach to prevent the hypersensitivity reaction, these molecules are not those generally prescribed when a respiratory exacerbation occurs and are not to be considered as a first line intervention in acute settings.

Most of the times, CF allergic patients may benefit from desensitization (DSS) protocols (Figure 2 and Figure 3). DSS is a therapy that allows acquiring drug tolerance. This process is thought to reduce the responsiveness of mast cells to the agent and/or neutralize specific drug IgEs, but the exact

mechanism by which immune tolerance is achieved is yet unknown (32). DSS has to be performed after obtaining a written informed consent from the patient. During the procedure, patients may develop severe allergic reactions. It seems that 76% of DSS procedures are effective (33). Nevertheless, since the effectiveness of DSS is limited to the duration of exposure to the drug (during a course of antibiotics), patients often require multiple DSS procedures for the same drug over time, every time the treatment in newly prescribed. DSS protocols are standardized for a few drugs only, but in Literature many protocols have been proposed both for immediate and delayed reactions (5, 34-38).

The intravenous route is preferable to the oral administration of the drug, since it allows a better control of the administered dose, and it may be stopped immediately whenever an allergic reaction occurs. Moreover, the injected dose is not modified by intestinal malabsorption, which is typical in CF patients suffering from pancreatic insufficiency. At last, it has to be underlined that most antibiotics that are active against P. aeruginosa are available intravenously.

CONCLUSIONS

Prognosis in CF patients is related to the respiratory disease, and respiratory failure is the leading cause of death. The significant increase in survival rates is largely due to better antimicrobial strategies. Over the past 40 years, the overall survival of CF patients has considerably improved, thanks to the possibility to be assisted in specialized centers, to the improvement in respiratory physiotherapy techniques, but also to the development of new antibiotics and therapeutic strategies, and to an optimized nutritional support. Hypersensitivity reactions to antibiotics are relatively common in patients with cystic fibrosis. The type of the reactions and the determining immunological mechanisms are not different from those reported by the general population. Beta-lactams are the antibiotic class most frequently involved in these reactions. There are several risk factors for developing allergic responses in CF patients, including an increased use of chemotherapeutic agent, which is related to an increased duration of life. Since antibiotic choice is often limited by the pattern of resistance of the pathogens, managing respiratory exacerbation may sometimes become difficult. It is therefore essential to classify the nature of the hypersensitivity reactions and to determine whether CF patients are allergic or if the reaction was related to non-immunological mechanisms. Desensitization is a viable option in most allergic hypersensitivity reactions. In any case, whenever an allergic reaction has been proven, the patient should receive an allergy card, clearly stating which drugs are proscribed, which alternative are possible, and which reactions has been previously recorded. Also, all allergic patients should benefit from an education on what to do in case of future reactions and how to promptly recognize them (Figure 2, Figure 3 and Figure 4).

CF specialists must have the reflex to ask the patient to consult a drug allergy unit in order to receive a proper and complete allergy work-up, and eventually to either search for useful alternative drugs or to benefit from a desensitization protocol.

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EMERGING AND FUTURE THERAPIES FOR ALLERGIC RHINITIS

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Allergic rhinitis (AR) is one of the most common diseases and represents a global health problem, currently affecting up to 30% of the general population, with a continuously increasing prevalence and significant comorbidities and complications. To date, the mainstay of current treatment strategies of AR includes allergen avoidance, pharmacotherapy and allergen-specific immunotherapy, as defined by Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines for both adults and children. The aim of this review is to provide an update on all emerging and future therapeutic options for the treatment of AR.

Allergic rhinitis (AR) is one of the most common diseases, currently affecting up to 30% of the general population, but its prevalence is steadily increasing both in children and adults (1). AR is actually a global health problem because of its prevalence and its impact on patients' social life, school performance, and work productivity (2). AR is considered a relevant and independent risk factor for developing asthma as it often precedes bronchial hyperreactivity (3, 4). Asthma and AR frequently coexist, with epidemiological data suggesting that up to 80% asthma patients also suffer from AR, and up to 40% of patients with rhinitis also have asthma (5). AR has also been linked to 'small airway disease', defined as a reduction in forced expiratory flow at 25–75% of the pulmonary volume and a normal spirometry, which is suggested to be an early marker of bronchial involvement in patients with AR who perceive only nasal symptoms (6). The link existing between the upper and lower airways has been recently defined as the concept of "united airways disease" (7), that has become also a cornerstone of ARIA guidelines with relevant implications for the diagnostic and therapeutic management of respiratory allergy, especially AR and asthma (1).

Symptoms of AR include rhinorrhea, nasal obstruction, nasal itching and sneezing, which are reversible spontaneously or with treatment (8). Allergic rhinitis symptoms result from a complex, allergen-driven mucosal inflammatory process, modulated by immunoglobulin E (IgE), and caused by interplay between resident and infiltrating inflammatory cells and a number of vasoactive and proinflammatory mediators, including cytokines (9-13).

The latest Allergic Rhinitis and its Impact on Asthma (ARIA) classification of AR is based on symptoms and quality-of-life parameters. Duration of symptoms is subdivided into "intermittent" or "persistent" disease, while severity is subdivided into "mild" or "moderate-severe", depending on symptoms and quality of life. Guidelines for the diagnosis and treatment of AR have already been

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published (1, 14); the mainstay of current treatment strategies of AR includes allergen avoidance, pharmacotherapy (particularly, antihistamines and intranasal corticosteroids as first-line agents) and allergen immunotherapy (AIT), as defined by ARIA guidelines for both adults and children (1). Evidence based guidelines, advances in drug treatments, and novel AIT have all improved the care of patients with AR, offering a wide array of pharmacotherapeutic options with relatively safe side effect profiles for the management of AR (15, 16). Many steps forward have also been made to improve outcome in the pediatric population (15).

The aim of this study is to review all emerging and future therapeutic options for the treatment of AR. We identified studies from MEDLINE database and reviewed the references used in the ARIA guidelines. References of the retrieved articles also were screened. We set no language restrictions.

EMERGING AND FUTURE THERAPIES

The current scientific research is aimed at identifying new therapeutic targets for the treatment of AR. This scientific effort mainly include discovery of new molecules related to current treatments and new insights in AIT and biological drugs; moreover, understanding of inflammatory mechanisms inspire ongoing studies with the purpose of modifying the immune response to allergens.

Oral H_1 antihistamines are currently the first line of treatment for acute symptomatic relief of AR, positively affecting all symptoms, with the expection of nasal obstruction (1, 17).

In order to reduce healthcare costs, the efficacy of a prophylactic treatment with a second-generation H_1 antihistamine has been recently explored. In a randomized double-blind study conducted by Yonekura et al, 50 patients with seasonal AR caused by Japanese cedar pollen who were randomized for treatment with levocetirizine hydrochloride 5 mg or placebo as follows: administration of placebo for 8 days (treatment A), single administration of levocetirizine on day 8 after placebo for 7 days (treatment B) or administration of levocetirizine for 8 days (treatment C) (18). Efficacy in each treatment arm was evaluated based on cedar pollen exposure for 3 h on day 9 in an enviromental challenge chamber, following 1-hour exposure on day 8. The total nasal symptom score on day 9, targeted as primary endpoint of the study, was significantly lower with treatment B compared with treatment A ($p < 0.01$). Treatment C did not show superior efficacy compared with treatment B. This study showed that early intervention with levocetirizine soon after onset of symptoms may attenuate these symptoms as effectively as prophylactic treatment before pollen dispersal (18). However, further studies are needed to examine whether other antihistamines have similar effects.

The identification of histamine H_3 and H_4 receptors has recently revived interest in histamine research and exposed attractive perspectives for the potential therapeutic exploitation of these new drug targets. While the H_3 receptor is mainly localised in the CNS, the H_4 is primarily expressed in hematopoietic cells, indicating their primary function in neurotransmission and immunomodulation, respectively (19). The evidence of histamine receptors H_3 has been recently demonstrated in human nasal tissue in close proximity of H_1 receptors, modulating vascular contractile responses and then disclosing potential applications also in allergic diseases (19).

Histamine H_3 receptor antagonists lead to an increase in norepinephrine and might have an advantageous decongestant effect in patients with AR administered with or without H_1-antihistamines, representing a significant advance in available treatments, with a potential superior safety and efficacy profile to the currently available oral decongestants (19).

PF-03654746, an highly selectively potent H_3 receptor antagonist, has been shown to reduce allergen-induced nasal symptoms, including obstruction, in combination with an oral H_1 antihistamine (fexofenadine) (20). Another novel selective H_3 receptor antagonist (JNJ-39220675), despite reducing nasal congestion in patients with allergen-induced AR, showed a limited potential antiinflammatory effect (21). More recently, the effect of PF-03654764 (an H_3 receptor antagonist with similar structure to PF-03654746) + fexofenadine on

subjective measures of allergen-induced congestion and Total Nasal Symptom Score (TNSS) was compared to pseudoephedrine + fexofenadine in a double-blind, placebo-controlled 4-period crossover study in patient with AR (22). The association of PF-03654764 + fexofenadine failed to provide superior relief of allergic rhinitis-associated nasal symptoms upon exposure to ragweed pollen compared to fexofenadine + pseudoephedrine; however, in post-hoc analyses, PF-03654764 + fexofenadine improved TNSS compared to placebo ($p = 0.001$) (22).

The histamine H_4 receptor is the newest identified member of the histamine receptor family. It is distributed mainly in hematopoietic cells, particularly mast cells, basophils and eosinophils. Preclinical data strongly suggest its potential therapeutic exploitation in inflammatory diseases such as AR, asthma, atopic dermatitis or pruritus (19). To date, none of H_3 or H_4 antihistamine is approved for clinical use, but some agents are in clinical trials for AR.

Combination therapy with different drugs, with different mechanisms of action, may provide a synergistic beneficial effects to selected populations of patients with moderate-to-severe symptoms. Recently, a novel fixed dose combination containing azelastine and fluticasone propionate has successfully been approved by the Food and Drug Administration (FDA) in 2012 and by European Medicines Agency (EMA) in 2013; it is licensed for the relief of symptoms of moderate to severe seasonal and perennial allergic rhinitis in adults and adolescents if monotherapy with either intranasal antihistamine or corticosteroid is not considered sufficient (23). Four randomized, double-blinded, parallel-group, placebo-controlled, multicenter trials sponsored by the manufacturer evaluated the fixed combination of intranasal azelastine 125 µg and fluticasone propionate 50 µg administered as one dose per nostril b.i.d. in patients with moderate-to-severe symptomatic allergic rhinitis ≥ 12 years of age. Three of the studies were published as a meta-analysis which found the fixed combination of azelastine and fluticasone propionate statistically significantly more efficacious in reducing baseline total nasal symptom score by 5.7 as compared to azelastine (4.4; $p < 0.001$), fluticasone propionate (5.1; $p < 0.001$) and placebo (3.0; $p < 0.001$). The findings were supported by secondary assessments of scores of specific nasal and ocular symptoms. Pharmacokinetic studies have revealed no interactions between the two active components (23). Further efficacy and quality-of-life studies of combination products of nasal antihistamines and corticosteroids are needed especially in primary care settings and in children before fixed combination treatment can be considered first line therapy in AR.

Some patients with AR refers a sensation such as the "wet feeling in the nose" and the "dripping down the throat" associated with aqueous intranasal corticosteroid formulations. To avoid certain sensory perceptions, "dry" intranasal corticosteroid formulations (beclomethasone and ciclesonide) have been recently received FDA approval, supported by evidence of efficacy and safety (24, 25).

AIT is considered the only disease-modifying treatment for AR considering its ability to alter the Th_2-biased immune response, while the pharmacotherapy acts only on symptoms (26-34). Randomised, double-blind, placebo controlled trials have demonstrated that both subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) improve the clinical symptoms of the disease and reduce drug consumption, showing a carry-over effect with long-lasting persistence of effects after discontinuation and preventing the onset of new sensitizations. They also reduce the risk of asthma onset (35, 36).

Research in immunotherapy continues in several directions. Standardization of allergen extracts to make available products with consistent potency has dramatically increased the reliability of commercially available extracts (37). The use of recombinant allergens or their mixtures also obtained results: a mixture of five recombinant grass pollen allergens showed effectiveness in reducing symptoms and the need for symptomatic medication in people with allergies to grass pollen. All treated subjects developed strong allergen-specific IgG1 and IgG4 antibody responses (38). In order to improve the target allergen to treat, many steps forward have been made thanks to the current availability of microarrayed recombinant allergens for diagnostic tests. Mostly in patients sensitized to profilins and other cross-reacting molecules, component-resolved

diagnosis might modify AIT prescription by improving the identification of the disease-eliciting allergen sources, especially pollens (37).

Investigators have also been studying adjuvants to improve efficacy of immunotherapy as well as modified allergens, either through chemical manipulation producing allergoids or directly producing recombinant proteins or significant peptides. Coupling with innate immune response modifiers, as for example Toll-like receptor (TLR) agonists, has disclosed an intriguing research landscape in the field of immunotherapy (39). In particular, a vaccine containing an adjuvant that act as agonist of TLR4 combined with ragweed pollen extract for the treatment of seasonal AR was found to be effective and well tolerated (40).

Furthermore, the addition of anti-IgE monoclonal antibody (omalizumab) to standard SCIT has been evaluated with promising results, improving the therapeutic efficacy and reducing the risks associated with immunotherapy (37). The use of omalizumab in addition to conventional SCIT in patients with seasonal AR and comorbid seasonal allergic asthma has been proved to be safe and efficient in reducing the symptom load during the pollen season (41). Considering the pediatric population, a randomized, double-blind, placebo-controlled trial involving 221 children with SAR was conducted with the aim to explore the additional antiallergic effect of omalizumab in combination with SCIT (42). The combination therapy with SCIT and omalizumab resulted in a significant reduction in rescue medication use (86%, $p = 0.001$) and number of symptomatic days (61%, $p < 0.001$); moreover, this combined treatment showed superior efficacy on symptom severity compared with SCIT alone (nasal symptoms: median 0.45 vs. 0.32, $p = 0.025$) (Rolinck). Furthermore, a safety analysis on the same population showed a good tolerability of SCIT + omalizumab (82% of patients) (43).

Finally, alternative methods of administration are still being studied for future use in clinical practice in order to improve adherence and tolerability of AIT. These approaches exploit the high density of dendritic cells and the low numbers of mast cells and basophils in skin and lymphnodes, which favors antigen presentation and minimizes side effects (26). Recently the intralymphatic and epicutaneous routes have been tested with promising results. A randomized controlled trial, including 165 patients with AR due to grass pollen allergy, was conducted to test safety, immunogenicity and efficacy of intralymphatic administration compared to SCIT. Intralymphatic immunotherapy was found to be safe and effective in ameliorating symptoms after only 3 injections, that was long lasting and comparable to that achieved after 3 years of conventional SCIT (44). Epicutaneous administration, with patches filled with grass pollen extract applied on skin, has proved to be safe, well tolerated and efficient in reducing symptoms than placebo in a placebo-controlled, double-blind trial in patients with AR (45).

Omalizumab is a recombinant humanized monoclonal antibody that reduces levels of circulating IgE and expression of IgE high affinity receptor (FCεRI) on mast cells and basophils (46). Its role in the therapy of allergic asthma and urticaria is well established (47, 48). Several ongoing trials will evaluate omalizumab efficacy in the treatment of other allergic diseases such as AR, as previously mentioned. Results from clinical trials demonstrated that omalizumab improved symptoms, quality of life and rescue medications needed in adults and adolescents (>12 years and above) with AR (49). In a recent meta-analysis of randomized clinical trials, the efficacy and safety of omalizumab was assessed in patients with poorly controlled AR. Of 11 clinical trials included in the meta-analysis, a statistically significant reduction in the daily nasal symptom severity score and in daily nasal rescue medication score was observed (49). According to this evidence, omalizumab may provide a new treatment strategy for AR. In particular, omalizumab may benefit patients with moderate-severe allergic rhinitis with proven allergen-specific antibodies who have no sufficient response to recommended medications (49). Moreover, treatment with omalizumab would be beneficial in patients with comorbid allergic rhinitis and asthma. The recently published position paper on pediatric rhinitis by the European Academy for Allergology and Clinical Immunology (EAACI) considers omalizumab as a possible treatment for

patients with AR and moderate-to-severe asthma, when other recommended therapies are ineffective (14). However, the use of omalizumab for the treatment of AR has not been approved and the high cost limits this application. Nevertheless, its periodic off-label use may be justified in patients with severe disease not responding to standard treatments (50).

TLR-mediated signaling induces proinflammatory responses and can both suppress and exacerbate allergic responses in the airways. TLR targeting may arrest the disease progression of an allergic response either by induction of tolerance to allergens and/or by redirecting the immune response away from the airways (39). As mentioned before, TLR agonists, such as TLR4 and TRL9, have been widely employed as adjuvants in some SCIT formulations, showing strong immunomodulatory effects (39). Intranasal administration of TLR7 agonist (AZD8848) and TLR8 agonist (VTX-1463) has revealed efficacy in the relief of symptoms in AR patients. Indeed, most of our knowledge regarding TLRs agonists/antagonists are from experimental studies, even if the first results hold considerable promise (39).

Considering the role of cytokines in the pathogenesis of allergic inflammation, the inhibition of their functions may be useful in the treatment of AR. IL-33, which is a member of the IL-1 family of cytokines, has the capacity to induce Th_2 cytokines production in Th_2 cells, mast cells, basophils and eosinophils, suggesting a potential role in Th_2 cytokine-mediated allergic inflammation (51). Serum levels of IL-33 are significantly increased both in animal models and in humans with AR. In an experimental animal model of AR, anti IL-33 antibody significantly reduced the nose-scratching events, ameliorated skin denudation and decreased the numbers of eosinophils in bronchoalveolar lavage (52). IL-17 and IL-23 are both cytokines predominantly involved in Th_{17} cells pathway; they have currently been associated with the development of various inflammatory diseases, such as psoriasis, arthritis and inflammatory bowel disease. It has been recently established a more pronounced pathogenic role for IL-23 only in a mouse model of AR, but their immunomodulatory effect in the pathogenesis of allergic diseases is still controversial (16). Suplatast tosilate is a novel immunomodulatory compound actually available in Japan, acting as antiallergic-selective Th_2 cytokine inhibitor. Its mechanism of action is currently under investigation, even if recent studies highlighted an inhibitory role on the GATA-3/IL-5 signaling pathway (53). Unmet clinical needs for current pharmacotherapy of AR are related to the fact that many patients may still suffer some residual symptoms despite best available treatment. It has been hypothesized that residual symptoms could be due to allergic mediators directly affecting nasal neuronal hyperresponsiveness by activation of the ion channel transient receptor potential vanilloid 1 (TRPV1), which is considered a mediator of itch in AR (54). Unfortunately, a highly selective and potent intranasal formulation of a TRPV1 antagonist (SB-705498) failed to demonstrate its clinical efficacy, suggesting a redundant mechanism of action for this class of compounds (54).

Interest in inflammation after allergen exposure has recently shifted attention on different mediators than histamine and leukotrienes. Prostaglandin D2 (PGD2), an arachidonic acid metabolite, has proinflammatory effects through interactions with the chemoattractant receptor homologous molecule on Th_2 cells (CRTH2), a 7-transmembrane type G protein–coupled receptor selectively expressed on Th_2 cells, T cytotoxic type 2 cells, eosinophils and basophils. CRTH2 mediates chemotactic activity by recruiting and activating TH2 lymphocytes and eosinophils and therefore it plays an important role in AR (16). A recent preliminary trial demonstrated the efficacy of oral CRTH2 antagonist (BI 671800) as a treatment for seasonal AR with a favorable safety profile (55). Another CRTH2 antagonist, named OC000459, has been not only demonstrated to promote nasal and ocular symptoms in allergic subjects exposed to grass pollen, but also to reduce airway inflammation and improve lung function in moderate persistent asthma (56).

There is evidence that adenosine plays a role in the pathogenesis of asthma and rhinitis. Elevated levels of adenosine have been found in bronchoalveolar lavage fluid and exhaled breath condensate of patients with allergic asthma. Increased formation of adenosine can also occur in chronically inflamed upper airways

as recently observed in exhaled breath condensates from patients with AR (16). Unfortunately, no recent studies are available regarding selective agonist or antagonist for adenosine receptor subtypes, even if they may represent valuable targets for drug development in AR (16, 57). No progress has also been made with regard to other promising molecules, such as rofleponide palmitate, b-tryptase and phosphodiesterase-4 inhibitors (16).

Epidemiological studies and recent experimental research support the idea that microbial stimulation of the immune system can influence development of tolerance to innocuous allergens (58). In several *in vitro* and *in vivo* studies, it is reported that probiotics can determine a wide array of immune effects potentially in contrast with the atopic constitution. Deficient exposure to these microbes might explain the increase in immunological disorders. The mechanisms of action of probiotics in respiratory allergy have been extensively described (58). In particular, it has been demonstrated that probiotics trigger the production of IFN-γ by blood leukocytes. Moreover, ingestion of probiotics enhance IL-12 production by blood lymphocytes after stimulation of Tcell mitogens. All these effects lead to a higher Th_1/Th_2 ratio, promoting the shift to Th_1-type immunity, thus favoring the suppression of Th_2-induced allergic inflammation (58). All these data make the gastrointestinal microbiota composition of particular interest as it provides a major source of immune stimulation and that seems to be a prerequisite for the development of oral tolerance.

A meta-analysis described the results of 12 randomized clinical trials with probiotics in AR, showing some improvements in clinical symptoms and in quality of life, and suggesting a beneficial effect of the consumption of specific probiotic strains belonging to the *Lactobacillus casei*, *Bifidobacterium longum*, *Bifidobacterium lactis* or *Lactobacillus paracasei* species (59). Although immunological changes on allergic inflammation of nasal mucosa in individuals affected by AR have been demonstrated after probiotic supplementation, the evidence of a beneficial effect of probiotics on allergic respiratory symptoms is still conflicting and their routine use as an additive therapy in patients with AR cannot be recommended (59).

CONCLUSION

AR represents today a global health problem, currently affecting up to 30% of the general population, with a continuously increasing prevalence and significant comorbidities and complications. Clinical practice guidelines have been implemented over the past 15 years, better improving the care of patients with AR and offering a wide array of pharmacotherapeutic options with relatively safe side effect profiles for the management of AR. Many steps forward have also been made to improve outcome in the pediatric population. Despite considerable progress in therapeutics, to date, the mainstay of current treatment strategies of AR includes allergen avoidance, pharmacotherapy and allergen-specific immunotherapy, as defined by ARIA guidelines for both adults and children. Possible treatment options in a near future coul be represented by combination therapy, based on preparations containing intranasal antihistamines and corticosteroids, and by immunological treatments including omalizumab, anticytokine agents and TLR stimulators.

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EXHALED BREATH TEMPERATURE IN ASTHMATIC CHILDREN

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Asthma is characterized by chronic inflammation of airways. Currently, no traditional method allows an easy daily evaluation of the degree of airway inflammation. Measuring inflammatory biomarkers in the breath is a very attractive approach to monitor asthma inflammation. In recent years, the measurement of exhaled breath temperature (EBT) has been proposed as a method capable of detecting the inflammatory status of the airways. The objective of this study is to strengthen the role of EBT in the diagnosis and monitoring of asthma. The study sample was represented by a group of 40 patients, of both sexes, aged 6-15 years. The elective criteria for submitting patients to EBT determination were abstaining from drugs in the preceding 24 h, fasting for at least 2 h, physical resting for at least 30 minutes, a body temperature between 35-37°C. The temperature in the room of the surveys ranged from 18 to 25°C. The EBT values of asthmatic patients were higher [(median (IQR): 29.77°C (30.67°C to 29.38°C) range 28.46°C min-max 34.78°C] than those of non-asthmatic ones (median (IQR): 28.22°C (29.09°C-27.7°C), range 27.09°C min-max 30.07°C] and this difference was highly significant (p <0.001). Furthermore, no significant difference was found between the EBT values of the following groups of patients: those exposed and not exposed to passive smoking, those receiving and not receiving leukotriene drugs, those receiving and not receiving specific immunotherapy, monoallergic patients and poliallergic ones, those sensitized and not sensitized to house dust, perennial allergic patients and seasonal allergic ones. In addition, the evaluation of the correlation of EBT values with body temperature (r = 0.119, p = 0.464) and ambient temperature (r = -0304, p = 0.057) did not show any significant correlation. Finally, no statistically significant correlation was demonstrated between EBT values and FEV1 (r = -0055, p = 0.81, Fig. 4). In conclusion, the data of the present study further support the hypothesis that EBT can be considered a good method for monitoring asthma.

Asthma is a multi-factorial disease, caused by the interaction between genetic and environmental factors: the first confers the susceptibility to the disease, the second acts as triggers of symptoms, respectively (1-3). Despite the clinical spectrum of asthma being highly variable, chronic inflammation of airways is always present, even though the symptoms are episodic; in fact, even in asymptomatic patients, it is possible to find specific signs of inflammation of the bronchial mucosa (4). In this regard, both in the diagnostic phase and in the monitoring of asthma, it would be useful to associate the evaluation of the airway inflammation degree (5) with the clinical and instrumental assessment of lung function (6-11). Currently, in asthmatic patients, no traditional method allows an easy daily evaluation of

Key words: asthma, children, inflammation, markers, exhaled breath temperature

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the degree of airway inflammation in hospital and/or at home. Measuring inflammatory biomarkers in the breath is a very attractive approach to monitor asthma inflammation, because it is non-invasive and it makes repeated sampling possible. However, there are important issues about reproducibility, variability, and sensitivity that need to be addressed before this approach can be recommended as an outcome measurement (12-14). In fact, it is known that in asthmatic patients the vasodilatation occurring during asthma exacerbations and the increased bronchial vasculature linked to the remodeling processes could influence the EBT (15). The objective of this study is to strengthen the role of EBT in the diagnosis and monitoring of asthma, illustrating the ease and speed of use of the modern devices available for its measurement.

MATERIALS AND METHODS

The study sample was represented by a group of 40 patients, of both sexes (24 males and 16 females), aged 6-15 years. Children under 6 years of age were excluded from the study, due to the need for active participation in EBT measurement and the difficulty of establishing a diagnosis of asthma under that age. The group of asthmatics (n = 20) consisted of 14 males and 6 females, the group of non-asthmatic (n = 20) of 10 males and 10 females. The diagnosis of asthma could be an old diagnosis or a new diagnosis of asthma performed according to the GINA guidelines at the time of the visit at our outpatient clinic. In this case, we proceeded, first, providing a complete medical history and clinical evaluation of the patient, then, we performed skin prick test to assess the presence of allergy and spirometry examination to determine the lung function. Moreover, all patients' parents were invited to answer the *ISAAC questionnaire* regarding the signs and symptoms observed in their children; finally the children exposed to passive smoking was assessed. The elective criteria for submitting patients to EBT determination were: abstaining from drugs in the preceding 24 h, fasting for at least 2 h, physical resting for at least 30 min, a body temperature between 35-37°C. The temperature in the room of the surveys ranged from 18 to 25°C. The device used for measuring the temperature of the exhaled air was *X-HALO®- thermometer for asthmatics – Delomedica*; its shape and size are similar to those of a glass and it is equipped with a mouthpiece, through which the patient has to breathe in tidal volume for a few minutes, as long as the device display shows the test result. However, the results can be downloaded onto a computer via the USB port, displaying the air temperature curve. EBT measurements were accompanied by the simultaneous registration of body temperature (by a tympanic thermometer *GENIUS TM2 Covidien)* and ambient temperature (by a portable thermometer *CK-10W Multimedia Brondi).*

Statistical analysis

The characteristics of the subjects and the results of the measurements were summarized using descriptive statistics. The qualitative variables (gender, exposure to passive smoking) were expressed as absolute frequencies and percentages, while the quantitative ones (age, EBT, ambient temperature, body temperature) as mean and standard deviation (SD) for normally distributed data or as median and interquartile range (IQR 75°-25°) for not normally distributed data. The evaluation of the type of distribution of the quantitative data was performed using the Kolmogorov-Smirnov normality test. The differences between the two study groups were evaluated using the Chi-squared test with Yates correction or the Fisher exact test for qualitative variables, using the nonparametric U Mann-Whitney test for quantitative variables. The correlations between EBT and body temperature, the ambient temperature and the FEV1 were evaluated using the Spearman's correlation coefficient. An α value less than 0.05 was used as the level of statistical significance.

RESULTS

The EBT values of asthmatics patients were higher [(median (IQR): 29.77°C (30.67°C to 29.38°C) range 28.46°C min-max 34.78°C] than those of non-asthmatics ones (median (IQR): 28.22°C (29.09°C-27.7°C), range 27.09°C min-max 30.07°C] and this difference was highly

significant (p <0.001, Fig. 1). From the evaluation of the relationship between exposure to secondhand smoke and EBT we found that the non-exposed subjects had a mean value of EBT (29.02±1.23°C) lower than the exposed ones (29.74±1.67°C), but this difference was not statistically significant (p> 0.05). Even asthmatic patients receiving leukotriene drugs showed a median value of EBT slightly lower than asthmatic patients not treated with the same drugs [respectively, EBT 29.41 (30.56-29.19°C) and EBT

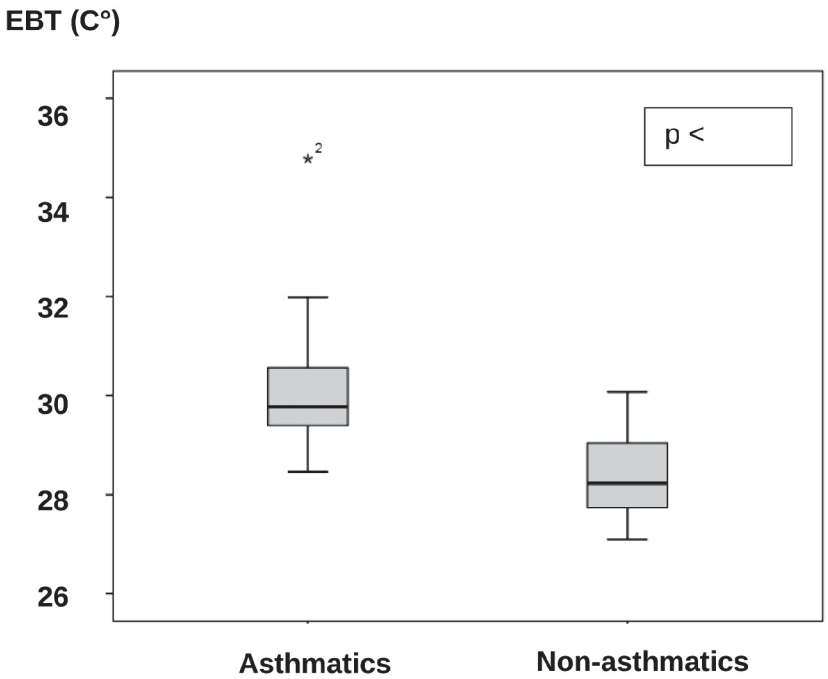

Fig. 1. *EBT values in asthmatic and non-asthmatic children.*

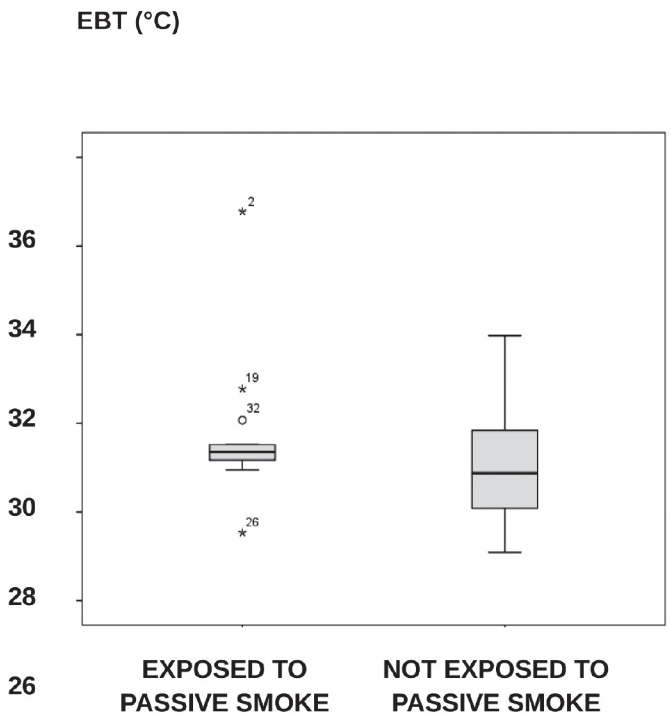

Fig. 2. *Correlation between EBT and Immunotherapy and between EBT and Leukotriene antagonists*

29.86 (30.78-29.52°C)], but even in this case, such a difference was not statistically significant (p> 0.05, Fig. 2). Moreover, no statistically significant difference (p> 0.05) was detected between EBT values of immunotherapy-treated allergic asthmatic subjects and EBT values of non-treated ones (EBT 29.30.19±1:46°C in the former and EBT 29.74±0:22°C in the latter) (Fig. 2). Furthermore, no significant difference was found between EBT values of patients positive to perennial allergens (mite and ragweed pollen) [29.73 (30.19-29.36°C)] and EBT values of patients positive to seasonal ones [31.06 (32.92- 29.42°C)] (p> 0.05, Fig. 3). By analyzing the relationship between EBT values and positivity to the various allergens, a different EBT was detected between mite-positive subjects EBT 29.41 (29.69-29.28°C) and mite-negative ones EBT 30.03 (31.06-29.73°C); however, this difference cannot be considered statistically significant, even if the *p value* is slightly above the significance limit (p = 0.07, Fig. 3). Also the comparison of EBT values between patients positive to more than one allergen [EBT 29.45 (30.11-29.31°C)] and patients positive to only one allergen [EBT 30.33 (31.99-29.82°C)], showed no statistically significant difference (p> 0.05, Fig. 3). The results of the study are summarized in Table I. In addition, the evaluation of the correlation of EBT values with body temperature (r = 0.119, p = 0.464) and ambient temperature (r = -0304, p = 0.057) did not show any significant correlation. Finally, no statistically significant correlation was demonstrated between EBT values and FEV1 (r = -0055, p = 0.81, Fig. 4).

DISCUSSION

In patients with asthma, inflammation is a constant feature, even in the absence of symptoms (16). Clinically, this phenomenon leads to an increased susceptibility of the airway mucosa to the environmental factors, with periodic exacerbations determined by the release of the mediators of acute inflammation. Anatomically, however, this phenomenon hesitates in the respiratory tree remodeling, through a vicious cycle characterized by the appearance of pathological repair processes, the secretion of growth factors, the proliferation of smooth muscle tissue, mucosal metaplasia, angiogenesis and airway fibrosis (17-19). In spite of the inflammatory nature of asthma, in clinical practice, the diagnosis and management of this disease is based on clinical history, evaluation of symptoms, physical examination and pulmonary function tests. The reason stems from the lack of simple methods to assess the degree of airway inflammation. Currently, the gold standard for the measurement of airway inflammation is bronchoscopy with biopsy and/or broncho-alveolar lavage, but this method is invasive and certainly not to be used as a routine practice, especially in children. The collection and examination of sputum is not feasible in all subjects and the process is pretty long (20). The measurement of the exhaled breath components as nitric oxide has shown some reliability, but it requires further standardization and cannot be used routinely for the high costs (21). However, the possibility to have a standardized method for the daily monitoring of the inflammatory

Table I. *Median values of EBT in the different groups of patients.*

GROUPS OF PATIENTS	MEDIAN VALUES OF EBT (°C)
Asthmatic *vs* Non-asthmatic	29.77/28.22 (p<0.001)
Passive smoking- exposed patients *vs* non-exposed ones	29.74/29.02 (p>0.05)
Antileukotriene drugs non-treated patients *vs* treated ones	29.86/29.41 (p>0.05)
Immunotherapy non treated patients *vs* treated ones	29.74/29.30 (p>0.05)
Seasonal allergens *vs* perennial ones	31.06 /29.7 (p>0.05)
Mite negative *vs* mite positive subjects	30.03/29.41 (p>0.05)
Monosensitized patients *vs* polysensitized ones	30.33/29.45 (p>0.05)

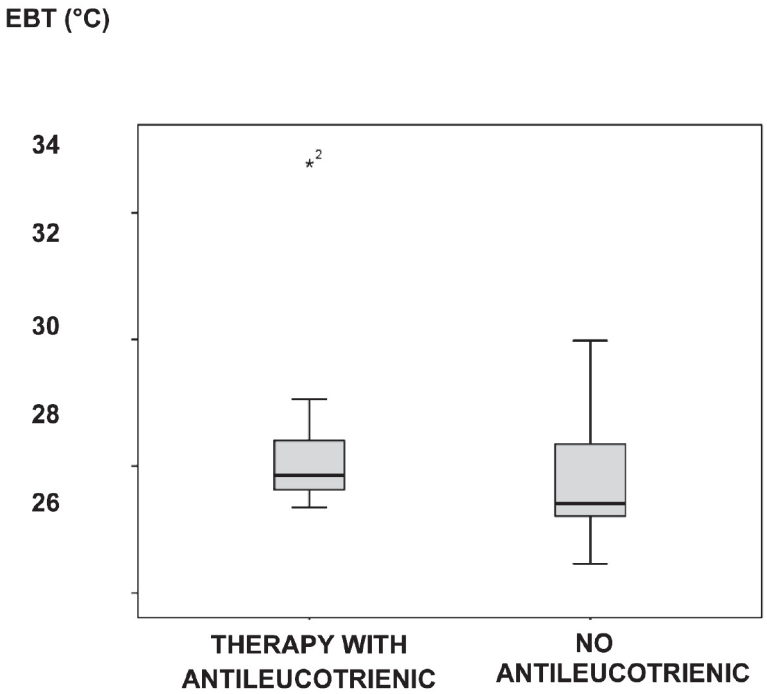

Fig. 3. *Correlation between EBT and mono/polisensitization, EBT and mite positivity/negativity and between EBT and seasonal/perennial allergen positivity.*

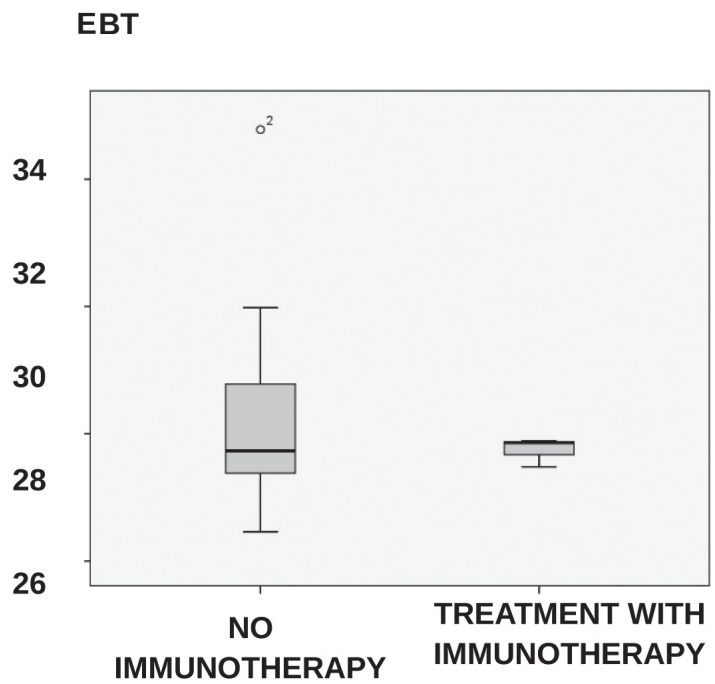

Fig. 4. *Correlation between EBT and Fev1.*

airway status would bring an easier detection of the asthmatic exacerbations in a pre-symptomatic stage, allowing the physicians to intervene therapeutically in more precocious times (6). In various tissues, inflammation provokes the classic signs of pain, heat, edema, hyperemia and loss of function. At least with regard to the increased production of heat and local hyperemia, inflammation seems to induce equivalent changes in the bronchial walls of patients with asthma (22). Although early studies about the physiology of the heat exchange of the respiratory tree date back to the 60s, studies involving direct measurements of the exhaled breath temperature were not conducted until the early 2000s. In order to investigate the hypothesis that *calor* may reflect airway inflammation, in 2002 Piacentini et al. (23) and Paredi et al. (24) published two different studies conducted by using the first prototypes for EBT determination. These studies showed the temperature curve of exhaled air according to two different approaches (respectively *EBT in final phase of expiration – PLET,* or *temperature increase rate - $\Delta e°T$*). For this purpose, the values of EBT were benchmarked against validated indicators of inflammation, such as asthma FeNO. A subsequent study by Piacentini at al. (25) showed that the control of asthma and the consequent reduction in EBT are correlated with a reduction of the eosinophil amount in bronchial secretion. A few years later, Popov et al. published a study that used a new tool for EBT measurement and a further different approach. This device was easier and more convenient to use than the previous one and was able to determinate the *maximal peripheral airway temperature* through repeated breaths at tidal volume. Even this method, similarly to the *PLET* and *$\Delta e°T$*, proved to be able to distinguish between asthmatics and non-asthmatics (25). Similarly to previous studies (26-27) aimed to check the validity of EBT as a marker of airway inflammation, our study has also demonstrated the ability of EBT to discriminate between asthmatics and non-asthmatics. There are no data in literature demonstrating the ability of EBT to discriminate, respectively, exposed and non-exposed to passive smoking, treated with leukotriene antagonists or with specific immunotherapy and non-treated subjects, perennial allergen positive subjects and seasonal allergen positive ones, poly-sensitized subjects and mono-sensitized ones. Our results revealed no significant difference of EBT values in the various tested groups; these data, however, could be affected by the reduced sample size. In agreement with the study conducted by Popov et al. (28), no correlation was found between EBT, body temperature and ambient temperature. Moreover, by analyzing the correlation between EBT values and FEV1, we found no significant correlation. This is in disagreement with the first findings by Paredi et al. (29), which showed a slight inverse correlation between the two parameters. The discrepancy of these results may, however, lie in the different method used for EBT measuring. In fact, the study published by Xepapadaki et al. (30), was performed by using a device similar to ours, showed no correlation between EBT and FEV1. The outcome of spirometry does not always correlate with markers of airway inflammation: inflammatory markers, in fact, provide information concerning the temporary status of inflammation, while spirometry cannot distinguish between airway remodeling and bronchial acute inflammation. In addition, it has been repeatedly shown that in asthma, lung function, inflammation and symptoms can vary independently. In conclusion, the data of the present study further support the hypothesis that EBT can be considered a good method for monitoring asthma. In fact, it provides similar and complementary information to other methods currently used to assess the inflammation level of airways. Indeed, the application of EBT measurement to other respiratory diseases has provided encouraging results about the potentiality of the method. From the early prototypes of the 2000s to the modern devices, handable equipment for EBT measurement is nowadays commercially available and already used in clinical practice. Thanks to the third generation devices it is also possible to obtain an immediate result which is not influenced by environmental factors. However, other studies are needed to define the reference values of EBT and its role in the diagnosis and monitoring of asthmatic disease.

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HIGH-MOBILITY GROUP BOX 1 IN ALLERGIC AND NON ALLERGIC UPPER AIRWAY INFLAMMATION

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High mobility group box 1, an evolutionary ancient protein conserved in the eukaryotic kingdom, exerts intra- and extra- cellular functions, orchestrating a homeostatic defensive response in challenged tissues. Its action associated with various inflammatory cells is essential for the occurrence, progression, and persistence of asthma, rhinitis, and nasal polyposis. The recent discovery of High mobility group box 1, as a critical mediator of inflammation, stimulated an increasing interest in the field of inflammation research, suggesting new therapies for atopic and non-atopic inflammatory processes.

High mobility group box1 (HMGB1), an evolutionary ancient protein conserved in the eukaryotic kingdom, exerts an intracellular nuclear function, with an important structural role and regulation of gene transcription, and an extracellular activity, secondary to a passive release from necrotic cells or an active production of macrophages, dendritic cells, and natural killer cells (1). HMGB1 is one of the most important damage-associated molecular pattern (DAMP) molecules, initiating and perpetuating immune response in non-infectious inflammatory processes (2). Its role, in fact, is to act as a 'danger signal', orchestrating a homeostatic defensive response in challenged tissues (3). Toll like receptors (TLR) 2 and 4 (4) mediate HMGB1 effects, such as the receptor for advanced glycation end-products (RAGE) (5). The pro-inflammatory properties of HMGB1, acquired upon its cellular release and consequent receptor stimulation, contribute to the pathogenesis of various human diseases (6-8). The recent discovery of HMGB1, as a critical mediator of inflammation, stimulated an increasing interest in the field of inflammation research, suggesting new therapies for viral/bacterial infection or for other inflammatory diseases (9, 10).

HMGB1 and upper airway inflammation

Airway inflammation is the main characteristic of asthma. The combined action of various inflammatory cells is essential for the occurrence, progression, and persistence of asthma, causing the hyper-responsiveness and airflow limitation exhibited by patients with the disease (11-15). The presence of neutrophilic inflammation in those with severe and fatal asthma suggests that the pathology of this type of asthma may be dependent on specific molecules, such as HMGB1. In fact, it was demonstrated that HMGB1-RAGE axis mediates allergic airway sensitization and inflammation in response to different allergens, amplifying the allergic response

Key words: High mobility group box 1, airway inflammation, atopy, children

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through eosinophil survival improvement and promoting the chemoattraction (16).

Moreover, HMGBl and RAGE levels were positively correlated with neutrophils, in induced sputum samples of asthmatic patients, with high levels assessed in severe asthma. Furthermore, levels of both proteins significantly decreased after asthma therapy, representing potential biomarkers of response to treatment (17). Moreover, a close relationship was also revealed between HMGB1 cellular localization and eosinophil infiltrate, highlighting an increased HMGB1 expression in the nucleus and in the intercellular space in allergic patients (18). The study of this molecule may lead to the identification of a new diagnostic biomarker in allergic diseases and it could open important scenarios for novel therapeutic strategies. In particular, anti-HMGB1 antibodies were tested in murine asthmatic models, demonstrating a reduction of immunoglobulin E, inflammatory cell accumulation, airway hyper-responsiveness, mucus synthesis, smooth muscle thickness, suggesting that blocking HMGB1 activity may reverse airway remodeling by suppressing airway inflammation and modulating lung fibroblast phenotype and activation (19). However, the airway epithelial barrier has been shown to be involved in different chronic disorders other than asthma (20, 21), including rhinitis (22, 23) and nasal polyposis. Chronic rhinosinusitis (24) with or without nasal polyposis, a clinical syndrome characterized by persistent inflammation of the mucosa of the nose involving secondly the sinus mucosa, results from a defective or excessive immune response to foreign agents with persistent inflammatory cell influx. It was demonstrated that HMGB1 was over-expressed in sub-epithelial infiltration and in the inflammatory cells, especially eosinophil cells, of these patients, suggesting its possible pathogenetic role in this chronic upper airway disease (18, 25, 26). In addition, HMGB1 was also assessed in nasal lavage of children affected by allergic rhinitis, demonstrating higher levels than controls and its close relationship with symptom severity (27), similarly to other inflammatory cytokines involved in the clinical expression of rhinitis (28). According to current knowledge, HMGB1 represents a common causal agent for various types of diseases and may serve as a future target molecule potentially useful for understanding disease processes. Future studies will define the pathways for HMGB1 release in disease, elucidate the most effective strategies for blocking the effect of this mediator, and determine the nature of any side-effects that develop when HMGB1 levels are reduced. In fact, certain substances, able to inhibit the release or inactivate HMGB1 protein, are under investigation. The study of HMGB1 should open new scenarios for diagnostic biomarkers and therapeutic strategies, both in childhood and in adult patients.

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ALLERGIC RHINITIS AND ADENOID HYPERTROPHY IN CHILDREN: IS ADENOIDECTOMY ALWAYS REALLY USEFUL?

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Allergic rhinitis (AR) and adenoid hypertrophy (AH) are common in children and are often associated with each other. Recent studies have shown improvement of respiratory symptoms and reduction in the adenoid volume after anti-allergic medical therapy (intranasal corticosteroids, antihistamines). The aim of our retrospective study is to evaluate the effectiveness of adenoidectomy on respiratory symptoms in pediatric patients with AR. We recruited 404 pediatric patients with AR, and we divided them into 4 groups (1. intermittent-mild rhinitis; 2. intermittent-moderate/severe rhinitis; 3. persistent-mild rhinitis; 4. persistent-moderate/severe rhinitis), using ARIA classification. For each patient we evaluated: age at onset of AR; family history of allergy; the presence of other allergic diseases; serum total IgE values; skin prick test (SPT) results; presence of AH evaluated by rhino-laringeal fibroscopy; adenoidectomy and its efficacy on respiratory symptoms. Our data show an association between AR and AH: 90 of 404 (22%) children with AR had AH of a degree greater than 2nd. A significant percentage (80%) of children suffering from AR did not present satisfactory benefits from adenoidectomy. They reported persistence or recurrence of rhinitic symptoms after surgery or only partial benefits, especially of recurrent respiratory tract infections and nasal obstruction. The local allergic persistent inflammation on nasal mucosa and adenoid tissue is probably the cause of the unsatisfactory results of adenoidectomy, therefore surgery can not be the first therapeutic step for these children. It is important to extinguish the local inflammation by medical anti-allergic therapy to obtain improvements of nasal symptoms and to prevent adenoid regrowth.

Allergic rhinitis (AR) and adenoid hypertrophy (AH) are common diseases in children, both with a great impact on the quality of life and often associated with each other. Adenotonsillectomy is the commonest surgical operation in children. The adenoids are immunosecretory organs, part of Waldeyer's ring or nasopharynx-associated lymphoid tissue, that play an important role in the host defence against pathogens (1, 2). Recent studies on atopic children have demonstrated a local production of specific IgE, IgA and IgG in the adenoid tissue of these patients and the presence of eosinophilic inflammation, which may be the cause or contributory cause (with recurrent respiratory

Key words: allergic rhinitis, adenoid hypertrophy, adenoidectomy, children

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infections) of nasopharyngeal inflammation and AH (3-7). Few studies have been conducted in order to assess the usefulness of adenotonsillectomy in patients with AR, considering as primary outcome the complete resolution of symptoms. More recent studies underline that a good clinical practice is to start with anti-allergic medical therapy (intranasal corticosteroids, antihistamines), because the greater part of patients, already after 3-4 weeks, shows an improvement of respiratory symptoms and a reduction in the adenoid volume (8-12). The aim of our study is to evaluate the frequency of AH and adenoidectomy in pediatric patients with AR and, above all, the effectiveness of adenoidectomy on the respiratory symptoms.

MATERIALS AND METHODS

Four hundred and four pediatric patients with AR participated in this retrospective study. They were aged between 4 and 17 years (median age 9 years) and are followed at the Pediatric Immuno-Allergology Department of the University Hospital of Messina. The adenoidectomy had been most commonly carried out in school age (6-10 years). We evaluated for each of them the severity of rhinitis, using ARIA classification that identifies: intermittent and persistent rhinitis, in relation to the duration of symptoms; mild and moderate/severe rhinitis, in relation to the severity of symptoms. We define intermittent a rhinitis with symptoms present for less than 4 days per week and for less than 4 weeks; while it is persistent when the symptoms are present for more than 4 days per week and for more than 4 weeks. The division into mild and moderate/severe rhinitis is carried out evaluating sleep quality, limitations of daily activities and reduction of school performance through questionnaires; the degree of the child's discomfort related to nasal symptoms, using VAS scale (visual analogue scale). We have thus divided the patients into 4 groups: 1) intermittent-mild rhinitis; 2) intermittent-moderate/severe rhinitis; 3) persistent-mild rhinitis; 4) persistent-moderate/severe rhinitis. Furthermore, for each patient we evaluated: the age at onset of the rhinitic symptoms; family history of allergy; the presence of other allergic diseases (asthma, persistent dry cough, conjunctivitis, atopic dermatitis, urticaria/angioedema, bronchus and/or laryngospasm); skin prick test (SPT) results; serum total IgE values; presence of AH evaluated by rhino-laringeal fibroscopy; adenoidectomy or adenotonsillectomy and their efficacy. The patients with typical rhinitic symptoms but with negative SPT and/or normal serum total IgE values were classified as suffering from Local Allergic Rhinitis (LAR). We also evaluated the presence of Recurrent Respiratory tract Infections (RRIs), using the criteria set by the Study Group of the Italian Society of Paediatric Immunology: more than six respiratory infections in 1 year; more than 1 high respiratory tract infection per month from September to April; 3 or more pneumonia or bronchopneumonia in 1 year.

RESULTS

The demographic, clinical, and bio-humoral characterists of enrolled patients are reported in Table I.

DISCUSSION

Our data show an association between AR and AH but the most important result is the evidence that a significant percentage (80%) of children suffering from AR did not present satisfactory benefits from adenoidectomy. In fact they report persistence or recurrence of rhinitic symptoms after surgery or only partial benefits, especially on RRIs and nasal obstruction. In any case, in these patients the efficacy of adenoidectomy can be considered absent or partial. The smaller group of patients with good response to surgery was characterized by mild rhinitis with negative family history of allergy in 4 out of 6 of them and RRIs before adenoidectomy. It is probable that in these patients the main causative factor for AH is the recurrence of infections in the high respiratory tract, that causes a continuous inflammatory state of the adenoid tissue and its consequent hypertrophy. In the second, greater, group of children without benefit after surgery, the main contributory cause of AH is AR. In fact, they had moderate/severe

rhinitis, positive family history of allergy, positive SPT (83% of cases), higher total IgE values and allergic comorbidities. The association between AR and AH was already known in the recent literature (1, 13, 14). The association between AR and AH can be explained by other recent studies that show a local production of IgA, total IgE and Dermatophagoides pteronyssinus (DP) and Alternaria-specific IgE in adenoid tissue of atopic patients, that cause local eosinophilic inflammation and AH (3, 15). IgE synthesis was demonstrated also in nasal mucosa and continues even outside the pollen season in grass pollen-sensitive patients (16). In agreement with our data, some studies show a greater association between AH and AR with hypersensitivity to dust mites (3). Adenotonsillectomy is the most frequent surgical operation in the pediatric population. The absolute indication for adenoidectomy is severe airway obstruction with OSAS (obstructive sleep apnoea syndrome); relative indications are chronic nasal obstruction, recurrent/chronic adenoiditis, recurrent/chronic sinusitis, recurrent acute otitis media and chronic otitis with consequent hearing loss. Side effects of adenotonsillectomy include post surgical inflammation of the posterior pharynx and Grisel's syndrome (17). Regarding the efficacy of adenoidectomy on clinical symptoms of rhinitis (persistent nasal obstruction, rhinorrhea, nasal itching, alteration of sleep quality), few studies have been carried out, with conflicting results (18). Our data, in agreement with the latter two studies described above, report a larger percentage of children with moderate-severe rhinitis and poor response to adenoidectomy in terms of improvement of rhinitic symptoms. In patients with AR, a local minimal persistent inflammation is present related to continuous stimulation of allergens, to cytokine dysregulation (characterized by upper levels of IL4, IL5, IL6, IL10, IL 23 (19), (20-22), HMGB1 (23- 25) and lower values of IL2, IL 12 and INF-γ) and to the presence of inflammatory cell infiltrate (eosinophils, neutrophils) in the nasal mucosa. We hypothesized that atopy-RRI correlation might result from Persistent Allergic Minimal Phlogosis (P.A.M.P.). A potential mechanistic association of viral infections with development of atopic disease

Table I. *Demographic, clinical, and bio-humoral characteristics of enrolled patients.*

	Adenoidectomised children with Allergic Rhinitis	
	Benefit afteradenoidectomy	No benefit after adenoidectomy
N° patients	6	24
Males	2	14
Median toltal IgE values (UI/ml)	200	767
Positive SPT for:		
Dust mites	0	16
Ragweed pollen	2	10
Animal epithelia	0	8
Mold	0	4
Positive family history for allergy	2	20
RRIs	4	16
Coexisting allergic diseases:		
Asthma	-	6
Persistentdrycough	-	8
Conjunctivitis	-	4
Atopicdermatitis	2	4
Urticaria / angioedema	2	6
Bronchusand /orlaryngospasm	-	6
Groups of rhinitis symptom severity:		
Group 1	2	2
Group 2	-	8
Group 3	4	4
Group 4	-	10

was previously described (26). Several investigators detected that increased specific (anti-viral) and non-specific IgE (27) could be a link to viral infections, increased airway hyperresponsiveness, enhanced sensitization to allergen and atopic disease. Further data subsequently highlighted the involvement and importance of Th1, Th2 and Th17 cytokines (28). Therefore, it seems to be a synergistic interaction between viral infections and allergic sensitization, suggesting a "multilpe hit" model for induction of persistent phlogosis which in turn generates an exaggerated and/or impaired response to injury. In addition, individual, anatomic, genetic, and immunological characteristics are influencing the natural history of atopy-RRI in childhood (29-31). Additionally, in the epithelial cells of nasal mucosa of these patients, as well as in asymptomatic patients, there is an increased expression of intercellular adhesion molecule-1 (ICAM-1 /CD-54) in presence of allegen exposure. ICAM1 can therefore be considered a marker of allergic inflammation. ICAM1 is also the receptor for more than 90% of the rhinoviruses, that use it to penetrate and infect the nasal mucosa epithelial cells. This mechanism can explain the RRIs which are typical of patients with AR. Obviously the RRIs contribute to the minimal persistent inflammation of the nasal and upper airway mucosa (32-45). However, our patients with AR and AH, and children without benefits after adenoidectomy reported an improvement in nasal symptoms after therapy with nasal saline washes, INS and oral antihistamines.

In conclusion, our data show that the efficacy of adenoidectomy in children with moderate/severe AR is absent, partial or only temporary in a large percentage of patients. The presence of a local allergic persistent inflammation on nasal mucosa and adenoid tissue is probably the cause of the unsatisfactory results of adenoidectomy. For this, surgery can not be the first therapeutic step in these children. It is important to extinguish the local inflammation through medical anti-allergic therapy (nasal saline washes, INS and oral antihistamines), to obtain improvements of nasal symptoms and to prevent any adenoid regrowth.

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SERUM IMMUNOGLOBULIN IgE IN A PEDIATRIC POPULATION: A RETROSPECTIVE ANALYSIS

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Allergic sensitization is mediated by immunoglobulin E (IgE) and an increase of their total value is frequently used to complete a correct diagnosis of atopy. Serum IgE may be considered a typical biomarker for the allergic phenotype. The aim of this study was to evaluate total serum IgE, according to sensitizations, and to find a cut off to discriminate between atopic and non-atopic subjects. Seven hundred and ninety-five patients were enrolled in this study. Serum levels of total IgE were measured by a fluorescence immunoassay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) while specific IgE levels were measured by immunofluorometric assay (ImmunoCAP; ThermoFisher, Uppsala, Sweden). Both tests were expressed in kU/L, according to manufacturer's instructions. Results: A difference for total IgE, according to the gender, has been found (p = 0,0012) with higher values for males than for females. A correlation has been found between total IgE and specific IgE, even distinguishing the population in sensitized and non-sensitized. A statistically significant difference has been found according to the presence or the absence of sensitization (p < 0,0001) and also considering mono-sensitized and poly-sensitized patients (p < 0,0001). ROC analysis has been performed to define a cut off for total serum IgE, according to sensitization and to the type of sensitization (mono-sensitization or poly-sensitization). Finally multiple regression models have been performed to describe total IgE response (positive or negative) and to predict total IgE values. Since clinical limitations are well known, total IgE provide a useful aid to define atopy, allowing the clinician to carry out further investigations in patients with total IgE values beyond normal limits.

Allergic sensitization is mediated by immunoglobulin IgE and an increase of their total value is frequently used in the completion of a correct diagnosis in allergology. However, about 30% of patients with atopic manifestations may have normal levels of total IgE and, on the opposite side, the increased levels of IgE can be detected in non-atopic subjects.

Numerous non-allergic diseases would alter the values of total IgE, such as parasitic infections, immunodeficiency and some cancers. Considering the value of total IgE as the only parameter, it is no not enough to a correct diagnosis because many variables para-physiological may modify the absolute number such as ethnicity, age, smoking and environmental pollutants.

Reference values have been defined in 1981 by Zetterstrom et al. and they are still frequently used (1). In literature there are also more recent studies about IgE reference values (2, 3, 4, 5, 6, 7, 8).

Measurement of serum total IgE and allergen specific IgE may stably provide more standardized and quantitative results than skin prick tests (SPT). Even if SPT are the first level of allergy, their

Key words: total IgE, specific IgE, sensitization

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diagnosis reliability and interpretation rely on a variety of factors.

This study presented serum profiles of total IgE and specific IgE to 24 allergens, among a pediatric population.

The aim of this study was to evaluate total serum IgE, according to the presence of sensitizations, and to determine a cut-off to discriminate between atopic and non-atopic population.

MATERIALS AND METHODS

Patients

Seven hundred and ninety-five patients (459 males; 336 females; mean age= 7,8679 years, age range: 1–18 years) were evaluated at the Foundation IRCCS Policlinico San Matteo of Pavia from May 2012 to December 2013. Serum levels of total and specific IgE were measured in all patients. Sensitizations have been evaluated, for all patients, according to the result of specific IgE. The entire population has been also divided into sensitized and no-sensitized subjects.

Methods

Levels of total and specific IgE were available for all patients. Total serum IgE levels were measured by a fluorescence immunoassay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) and expressed in kU/L, according to the manufacturer's instructions. The serum concentration of total IgE is age-related: it increases during childhood until at about 10 years of age and it reaches values that are maintained during adult life. Specific serum IgE levels were measured by immunofluorometric assay (ImmunoCAP; ThermoFisher, Uppsala, Sweden) and expressed in kU/L, according to the manufacturer's instructions. Specific IgE levels higher than 0.35 kU/L were considered positive. For all patients, Specific IgE belonged to a standard screening pane containing 24 of the most common tropho-allergens and pneumo-allergens: *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Betula verrucosa, Corylus avellana, Egg white, Milk, Fish (Cod), Sesame seed, Peanut, Soybean, Hazel nut ,Tomato, Egg yolk, Walnut, Cat dander, Dog dander, Ambrosia trifida, Artemisia vulgaris, Parietaria officinalis, Cynodon dactylon, Poa pratensis, Alternaria alternate, Latex and Lysozyme.*

Statistical analysis

Statistical analysis was performed using the statistical software package Medcalc 9 (Frank Schoonjans, BE). Medians (md) and percentiles (25th and 75th, IQR) were used as descriptive statistics. The correlation between total serum IgE and specific serum IgE has been evaluated. The non-parametric Mann Whitney test was used to compare samples. In addition, a receiver operating characteristic (ROC) curve analysis was performed in order to determine a cut-off for total IgE that could optimize the sensitivity and the specificity of the test, to identify responder subjects. In addition multiple regression analysis was performed, respectively, to describe total IgE response and to predict total IgE values. The stepwise strategy was adopted to select significant independent predictors. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Measuring Specific serum IgE levels on the standard screening pane of 24 allergens, 275 (34,59%) patients resulted no sensitized while the remaining 520 (65,41%) were sensitized. The families of sensitization were classified into four principal groups: inhalant, food allergen, food recombinant and professional. Sensitized patients belonged from two groups: 188 children were mono-sensitized to one of the four sensitization's families and 322 were poly-sensitized to more than one.

Specific IgE showed the following values for median and percentiles: 6,46 kU/l (IQR: 0,91 - 82,865); total IgE showed the following values: 129 kU/l (IQR: 42,35 - 424,75). We initially compared total IgE concentrations by gender and we found that males had a greater median than females (150 kU/l in male; 99,45 kU/l; p = 0,0012). Then characteristics of population were analyzed on the basis of specific IgE response, dividing the distributions into no sensitized and sensitized subjects (Table I).

A correlation between total IgE and specific IgE

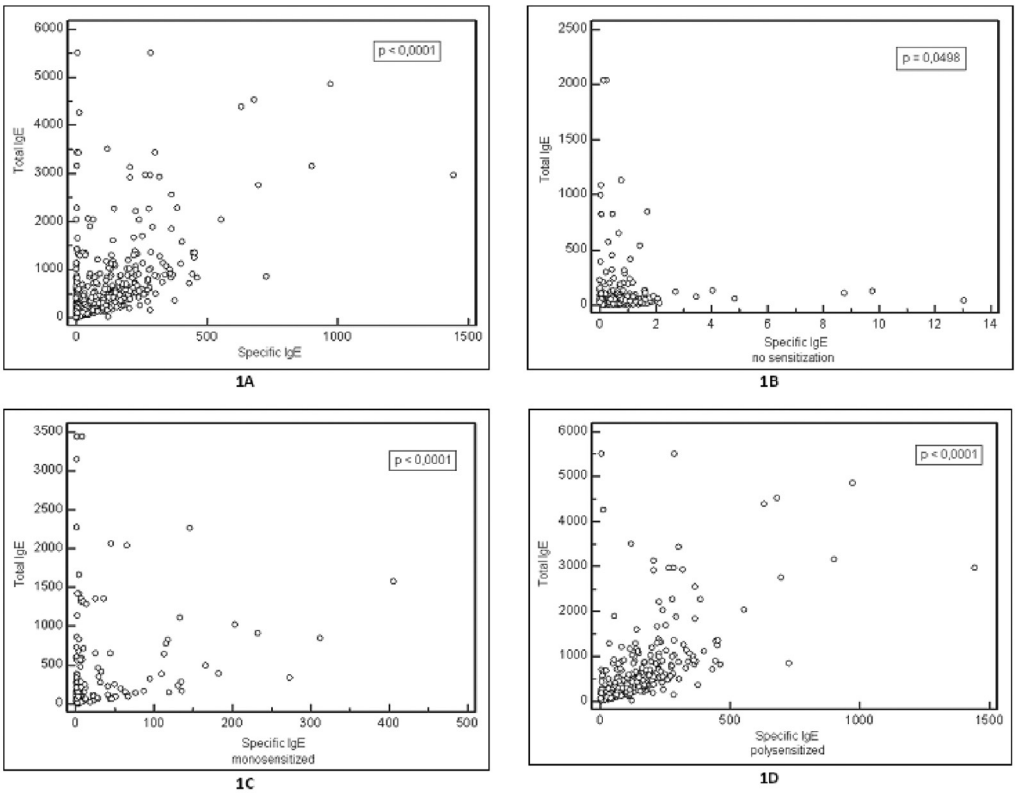

Fig. 1. *Correlation between was evaluated: correlation between total IgE and specific IgE in the entire population (A) and dividing total IgE distribution in no sensitized (B), mono-sensitized (C) and poly-sensitized patients (D).*

was observed in the entire population ($p < 0,0001$) and also dividing into the groups of no sensitized ($p = 0,498$) and sensitized ($p < 0,0001$). These results showed that there is a stronger association in sensitized subjects than in not sensitized. Therefore the correlation was studied dividing sensitized subjects into two subgroups: mono-sensitized and poly-sensitized. This sub analysis showed a strong correlation for both groups ($p < 0,0001$) (Fig. 1).

Considering total serum IgE values as divided in positive or negative result of specific IgE, a statistically significant difference has been showed ($p < 0,0001$). Particularly the analysis highlighted higher values of total IgE for sensitized patients. In addition the difference between total IgE, considering also the subgroups of mono-sensitized and poly-sensitized subjects, has been investigated. This deepening confirmed the significance obtained ($p < 0,0001$), stressing that total IgE values increase on the basis of sensitization. Poly-sensitized patients had higher values of total IgE than the mono-sensitized ones.

ROC analysis has been performed for total IgE, according to the presence or the absence of sensitization, and showed the best cut-off at 73,4 kU/L (Area Under the Curve = 0,861; Sensitivity = 84,2; Specificity = 76,4; $p = 0,0001$). Therefore another ROC analysis has been performed in order to find the best cut off to discriminate between mono-sensitized and poly-sensitized and showed a value of 166 kU/l (Area Under the Curve = 0,664; Sensitivity = 70,8; Specificity = 58; $p = 0,0001$) Fig. 2).

Two multiple regression analyses were performed: the first one with the aim to predict the result, positive or negative, of total serum IgE, the second one to predict the quantitative result of total serum IgE. In both models we also considered age, sex, sensitization (no sensitization, one or more sensitization) and sensitization's classification as independent variables. For the first model we considered the result, either positive or negative, of specific IgE, while for the second one we considered the quantitative result of specific IgE. Both models

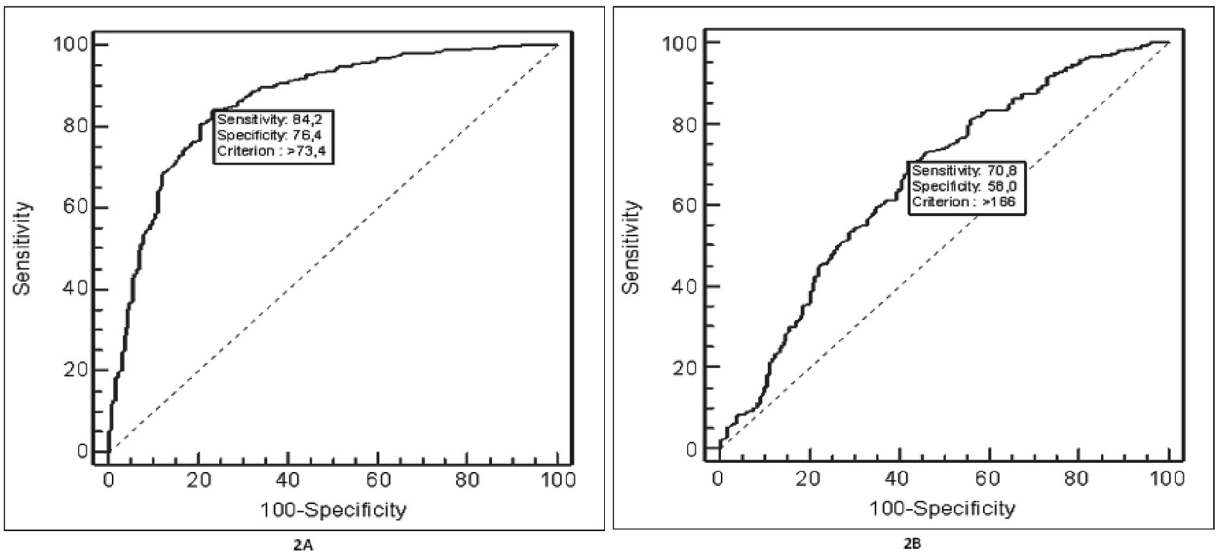

Fig. 2. *The Receiver Operating Characteristic analysis for total IgE, according to the presence or the absence of sensitization showed a cut off of 73,4 kU/L (A) while, according to the presence of one or more sensitizations, the cut off is 166 kU/l (B).*

resulted statistically significant (Table II). Specific IgE and sensitization's classification were predictors for both model while, for the first one, also sex and the type of sensitization were significant to predict the result.

DISCUSSION

Analysis of serum IgE concentration is usually used in daily allergological practice to obtain a correct diagnosis of atopy. Serum IgE may be considered a typical biomarker for the allergic phenotype, as allergic disorders are characterized by IgE-mediated inflammation.

A previous study, which considered serum IgE in a large cohort of allergic patients, demonstrated that total IgE values increase with the age, while specific IgE values decrease (9). Our study showed strong relation between total serum IgE and specific serum IgE. We did not consider the impact on age but the entire pediatric population has been divided into sensitized and no sensitized children.

Simple tests such as skin prick testing (SPT) and serum specific IgE testing are the most commonly used diagnostic tests to evaluate for IgE-mediated reactions (10, 11). While skin prick tests are the first level of allergy diagnosis, their reliability and interpretation rely on a variety of factors. Instead, measurement of serum total IgE and allergen specific IgE may stably provide standardized and quantitative results.

Our results underling a significant difference for total IgE levels to be higher in males than in females, like other studies reported (12). Genders also reflects a complex and diverse pathogenic role of IgE expression.

Our findings showed a significant variation of total IgE in sensitized patients than in no sensitized ones; particularly, values increased with sensitizations so that we obtained higher values in those patients who were poly-sensitized than no-sensitized ones. These findings suggested that a higher level of total IgE may be predictive of poly-sensitization to several allergens, according with a previous study (13).

The analysis showed an interesting result about the cut-off found: it may be useful a new valuation of reference values used in the clinical practice. Our data should be interpreted and reconsidered in relation to personal and geographic characteristics of the studied population, according to other previously studies because it is worth to note that para-physiological variables could determine a variation

Table I. *Characteristics of the studied population.*

	Non-sensitized (n=275)	**Sensitized (n=520)**	**p**
Age (years)	6.00 (4.00 – 9.00)	9.00 (5.00 – 12.00)	< 0.0001
Gender : male/female (No.of subjects (%))	145 (52.73%) /130 (47.27%)	314 (60.38%) /206 (39.62%)	0.0451
Total IgE (kU/l)	34.00 (12.025 – 71.875)	236.50 (104.50 – 646.50)	< 0.0001

All data are reported as median, with 25th and 75th percentiles in parentheses; P values refers to the Mann Whitney test, unless other specified. # p value refers to Chi-squared test

Table II. *Multiple regression models to describe total IgE response and to predict total IgE values.*

Dependent variable: Total IgE (positive or negative)				
Indipendent variables	Coefficient	Std. error	t	p
Constant	0.2781			
Sex	-0.06520	0.02878	-2.266	0.0237
Specific IgE (positive or negative)	0.1982	0.07111	2.787	0.0054
Sensitization	0.1153	0.03647	3.162	0.0016
Sensitization's family	0.05752	0.01015	5.667	<0.0001
Coefficient of determination R^2= 0.3364				
Significance level: P <0.001				

Age was not included in the final model because it did not achieve statistical significance as predictor.

Dependent variable: Total IgE				
Indipendent variables	**Coefficient**	Std. error	t	p
Constant	**71.5113**			
Specific IgE	**2.6922**	0.1754	15.348	<0.0001
Sensitization's family	**73.5007**	11.1536	6.590	<0.0001
Coefficient of determination R^2= 0.3866				
Significance level: P <0.001				

Age, sex and sensitization were not included in the final model because they did not achieve statistical significance as predictors.

in total IgE values (14). Therefore this study showed a way to determine the positivity or the negativity result of total serum IgE: sex, response to specific serum IgE, sensitizations and sensitization's family seem to be good predictors to total IgE result. In addiction we built a model to predict total serum IgE values: specific IgE values and sensitization's family seem to be good predictors.

Our aim is to continue and extend this study by the use of clinical aspects in order to determine how often sensitizations conduct to a real allergy. Since clinical limitations are well known, total IgE provide a useful aid to define atopy, allowing the clinician to carry out further investigations in patients with total IgE values beyond normal limits. In association with other tests, in vivo and in vitro, serum IgE concentrations could lead to a correct diagnosis.

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MANNITOL BRONCHIAL CHALLENGE TEST IN ASTHMATIC CHILDREN

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Bronchial asthma is a chronic inflammatory disease characterized by bronchial obstruction, usually reversible spontaneously or after therapy, bronchial hyperreactivity and accelerated decrease of lung function that may possibly evolve into irreversible obstruction of the respiratory tract. Bronchial provocation tests can be used in order to assess the presence and degree of bronchial hyper reactivity. The recently introduced mannitol powder inhalation indirect test seems to have an interesting and promising role, especially in childhood, because of its high diagnostic specificity, easiness of execution and best standardization. In this study the authors focused on the significance and clinical use of mannitol bronchial challenge test in asthmatic children.

Bronchial asthma is a chronic inflammatory disease characterized by bronchial obstruction, usually reversible spontaneously or after therapy, bronchial hyperreactivity and accelerated decrease of lung function, that may possibly evolve into irreversible obstruction of the respiratory tract (1). Asthma is clinically manifests by: dyspnea, cough, wheezing and chest tightness (1). The narrowing of the airways causes these symptoms, that may occur singly or in combination (1); even though their intensity varies in relation to bronchial obstruction's entity and the degree of perception threshold of the patient. (1) Asthmatic symptoms are often not specific, therefore lung function tests and new biomarkers of airway inflammation are being developed in order to improve diagnosis (2-8)

Bronchial provocation test

Bronchial provocation tests can be used in order to assess the presence and degree of bronchial hyper reactivity. These tests are divided into direct or indirect. The direct tests acting directly upon the bronchial smooth muscle. An example of direct method is Methacoline challenge test that stimulate muscarinic receptor in bronchial smooth muscle inducing bronchoconstriction (9). On the other hand, indirect tests are brought about indirectly through an effect of mediator release induces bronchial constriction. These tests are carried out with many methods, that are different according to the type of stimulus used, the pathophysiological mechanism of the response, the method of execution (9). The stimuli used in tests can be mechanical, physical or pharmacological. Examples of indirect tests are exercise induced bronchoconstriction (10), inhalation of adenosine monophosphate or mannitol (11).

The recently introduced mannitol powder inhalation indirect test, seems to have an interesting and promising role especially in childhood, because of its high diagnostic specificity, easiness of execution and best standardization (9). During this test, we induce bronchial obstruction through an indirect action on the bronchial smooth muscle fibers, by pro-inflammatory mediators and/or

Key words: mannitol; challenge test; asthma, children

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neuromediators issued by the effector cells, such as mast cells, eosinophils and neuronal cells. Compared to direct ones, indirect tests have less sensibility, but more asthma diagnostic specificity, due to better correlation with inflammation and with airways reactivity (11,12).

Mannitol test

In the end of 90's, Australian Anderson S.D. experimentally introduced the indirect bronchial provocation test with mannitol, as an alternative to hypertonic saline test, to assess exercise – induced bronchospasm in athletes with asthma (13). Mannitol is an organic crystalline glyco-polyol compound, extracted from the sap of Fraxinus Ornus (tree of "manna") and it is used in pharmacopoeia as osmotic diuretic (13).

Considering its relative harmlessness and its physical – chemical characteristics (hygroscopic powder), it proved to be suitable for encapsulation, and inhalation, then. In vitro studies revealed that mannitol can stimulate the realease of histamine from human mast cells. Mast cell degranulation is the main cause to induce exercise induced bronchospasm, mannitol bronchial provocation test has been subsequently introduced in clinical practice as a substitute for exercise test and hyperventilation isocapnica test with cold and dry air. The osmotic action of inhaled mannitol cause an increase in fluid osmolarity of the bronchial epithelium, so that water migrates from the cells to restore bronchial fluids, as a result, cells are reduced in volume (14) . Mediators of inflammation as prostaglandin (9, 11, PGF2), leukotrienes (LTE4) and histamine (l-metilistamina) are released which then act through specific receptors causing the contraction of bronchial smooth muscle and airway narrowing (14). The kit used in the test consists of mannitol dry powder in capsules in increasing doses (0 – 5 – 10 – 20 - 40 mg) and a disposable inhaler to inhale mannitol dry powder.

The test begins with a simple baseling spirometry, followed by the inhalation of an empty capsule (0 mg). During the inhalation, subjects must use nasal clips and exhale through the mouth to minimize the oro-pharyngeal deposition of mannitol powder (9,15). One minute after inhalation, it is rated the best FEV1 achieved in three spirometric measurements. The FEV1 achieved after 0 mg capsule, pre–challenge FEV1, is used to calculate the FEV1 percentage reduction in response to the further mannitol administration.

The test is positive for a mannitol dose that causes the fall of FEV1 > 15% compared to the baseline, or a >10% fall between two consecutive doses, until a maximum cumulative dose of 635 mg. The dose-response relation achieved from the FEV1 fall (%) is also considered in comparison with the cumulative mannitol dose that is necessary to cause this fall. Thus, it is possible to determine if the bronchial hyperreactivity of the patient is moderate, mild or severe: moderate for a mannitol dose < 155 mg; severe for a mannitol dose < 35 mg (14).

Subject affected by bronchial asthma are able to perform mannitol test after seven years of age. This test determines the level of bronchial hyperreactivity and the response to corticosteroids therapy too (13). Indeed mannitol sensibility is reduced, or even totally inhibited, after chronic administration of inhaled corticosteroids, thereby allowing to monitor the therapy. Moreover, in subject affected with asthma controlled by ICS (13,16), the mannitol test is used to forecast/predict exacerbations after the steroids dose titration (9).

CONCLUSIONS

Mannitol test is considered safe both in adults and in children, has high specificity, close correlation with inflammation of the respiratory tract, and it is useful to determine exercise - induced asthma. Furthermore the test is standardized, reproducible, and requires no special equipment. It is willingly accepted by the patient and approved by regulatory authorities.

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PRIMARY NOCTURNAL ENURESIS IN CHILDREN WITH ALLERGIC RHINITIS AND SEVERE ADENOTONSILLAR HYPERTROPHY: A SINGLE CENTER PILOT STUDY

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Nocturnal enuresis is defined as intermittent urinary incontinence during sleep that occurs at least twice a week for three consecutive months. There is no unifying etiology for nocturnal enuresis in the pediatric population and the disorder is likely to be multifactorial. We aimed to investigate the relationship between primary nocturnal enuresis, allergic rhinitis, and related complications in a paediatric case series from a single Center. We retrospectively reviewed and prospectively followed-up at our Institution (i) 32 children (14 females, 18 males; mean age 6.31±1.21 yrs) affected by allergic rhinitis with adenoidal hypertrophygrade I-II (group A) and (ii) 27 children (11 females, 16 males; mean age 6.52±1.33 yrs) affected by allergic rhinitis with adenoidal hypertrophy grade III-IV (group B). Allergic rhinitis was diagnosed on the basis of (a) typical nasal symptoms due to atopic sensitization (e.g., rhinorrhea , itching, sneezing fits, and nasal congestion and obstruction) and (b) positive skin prick testing and/or increased level of total serum IgE. We identified discrepancies between group A and group B in terms of risk of primary nocturnal enuresis. In fact, only 1 child of group A (3.12%) reported uncomplicated primary nocturnal enuresis; conversely, 6 children of group B (22.22%) showed a history of uncomplicated primary nocturnal enuresis (p=0.040). There was no statistically significant difference between the two groups in terms of atopic sensitization and serum total IgE levels (p=0.43). Allergic rhinitis may potentially influence the onset and the natural history of nocturnal enuresis in some children. Children with allergic rhinitis and more severe respiratory manifestations, seem to be more prone to developing primary nocturnal enuresis, likely due to potential multi-factorial causes (e.g., sleep disorders, chronic phlogosis, immune deregulation).

Nocturnal enuresis (NE) is defined as intermittent urinary incontinence during sleep that happens at least twice a week for three consecutive months (1). Generally, it occurs in a child before the fifth year of age. Enuresis can be classified as primary (urinary incontinence in a child who has never been dry) and secondary (urinary incontinence in a child who has been dry for at least 6 months) (2). The pathophysiology of primary NE (PNE) is still unclear but many authors have suggested many potential factors as cause, including the inability to awake from sleep in response to a filled bladder, excessive urine production rate during the night and/or decreased functional continence of the bladder (1). Therefore, etiology of PNE in the pediatric population appears to be multifactorial. Several conditions, including diabetes mellitus or insipidus, chronic kidney disease, neurological

Key words: nocturnal enuresis; atopy; allergic rhinitis; children

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and/or psychiatric disorders, and constipation have been associated with secondary enuresis (1, 2). Also paediatric obstructive sleep apnoea syndrome (OSAS) has been regarded as a potential co-factor in the onset and/or triggering of nocturnal enuresis. This respiratory disorder is more commonly due to an underlying adenotonsillar hypertrophy, and can be characterised by prolonged episodes of partial obstruction (obstructive hypopnoea) and/or complete obstruction (obstructive apnoea) of the upper airways (3, 4). Additionally, nasal chronic allergic inflammation has been reported as a major risk factor in predisposing to the adenoidal and tonsillar tissue hypertrophy. In this regard, several studies have so far addressed the beneficial effects of adenotonsillectomy in improving nocturnal incontinence in children with simultaneous allergic rhinitis and adenotonsillar hypertrophy, suggesting an etiological link between adenotonsillar hypertrophy, OSAS and nocturnal enuresis(5-9). In line with these findings, two case–control studies directly correlated the degree of adenoidal hypertrophy with manifestations of nasal atopy in the context of allergic rhinitis (10, 11). Moreover, an increase in adenoidal size during the pollen season in children with pollen-driven rhinitis has been also found (10). On the basis of the above findings, allergic rhinitis seems to have a key role in initiating and maintaining adenoidal hypertrophy which respectively lead to increased incidence of OSAS (11), OSAS-related sleep disorders and, possibly, nocturnal enuresis. The aim of the present study is therefore to investigate the relationship between PNE, allergic rhinitis, adenotonsillar hypertrophy, OSAS and enuresis.

MATERIALS AND METHODS

Subjects and experimental design

We evaluated 32 children (14 females, 18 males; mean age 6.31±1.21 yrs) affected by allergic rhinitis with grade I-II adenotonsillar hypertrophy and 27 children (11 females, 16 males; mean age 6.52±1.33 yrs) affected by allergic rhinitis with grade III-IV adenotonsillar hypertrophy. The degree of adenoidal hypertrophy was assessed by flexible nasal endoscopy using traditional tonsillar and adenoid volumetric indices for age. Children were recruited at the University of Messina, Department of Paediatrics. All the participating subjects were of Caucasian origin. The study was explained in detail to the parents/guardians of the children and an informed consent was obtained prior their inclusion in the study. Our Institutional Review Board approved this project.

The inclusion criteria were (i) age between 3 and 12 years; (ii) diagnosis of allergic rhinitis; (iii) presence ofadenotonsillar hypertrophy documented by ear, nose and throat (ENT) clinical examination and flexible nasal endoscopy; (iv) history of OSAS-related sleep disorders identified by nocturnal polysomnography test and/or questionnaires; (v) assessment of uncomplicated primary nocturnal enuresis with daily micturition diary (primary onset, normal stream, no or mild day-time frequency enuresis). Allergic rhinitis was diagnosed on the presence of typical nasal symptoms (nasal itching, sneezing, watery rhinorrhea, and nasal obstruction); sensitization to pollen allergen, when present, was documented by positive skin prick tests (SPTs), and/or increased level of total serum IgE (14). The severity of nasal symptoms was assessed using the Visual Analog Scale (VAS). The patients had to indicate nasal symptom severity through the VAS by putting a trait on a 10 cm segment. At the left end of the line (0 cm) symptoms were absent and at the right end (10 cm) severity was maximal (14). SPTs were performed using a panel of aeroallergens (including: house dust mite, mixed grass, Parietaria, birch, olive tree, Alternaria, cat, and dog; Allergopharma, Reinbek, Germany). Normal saline and histamine were used as negative and positive controls, respectively. Skin wheal diameter was recorded at 15 minus the mean of 2 perpendicular measurements. A positive response was defined as a skin wheal diameter of 3 mm or more compared to negative control (15). Total serum IgE levels were measured by ImmunoCAP100 system (Phadia, Uppsala, Sweden). The nasal and rhino-pharyngeal regions were assessed with a flexible paediatric endoscope. Adenoidal hypertrophy, depending on the rhino-pharyngeal space occupied,

was graded from 1 to 4, as follows: Grade 1, from 0 to 25% of the rhino-pharynx, Grade 2, from 25 to 50%, Grade 3, from 50 to 75% and Grade 4 > 75%. The nose was assessed for septal deviation, mucosal thickening and polyps. The oral cavity was examined for tonsillar enlargement, tongue and soft palate size and appearance. The size of the tonsils was graded by a scale from 0 to a maximum of 4 when the tonsils met at the midline (16, 17). Patients were evaluated by their medical charts, physical examination, laboratory tests and subsequent radiological evaluation (i.e., ultrasound bladder measurements) when necessary. Each child completed a micturition daily diary regarding voided volume, urge time, urge degree, incontinence time and amount of urine. For the purposes of this study, enuresis was defined as night-time bedwetting or daytime incontinence of any degree in children older than 3 years and already trained to use the toilet. Exclusion criteria were presence of associated diseases (genetic syndromes, immunodeficiency, congenital malformations, neurological and psychiatric issues; encopresis; history of urinary tract infections; diabetes mellitus); other nasal disease (e.g., structural abnormalities, nasal polyposis, clinically relevant septum deviation) and use of any treatment in the previous month capable of interfering with the results. Patients whose medical records did not have the necessary information for the study (i.e., i-v), and patients who had previously undergone adenoid and/or tonsil surgery were also excluded.

Statistical analysis

Statistical analysis and inter-group comparisons were performed with the Mann-Whitney U-test and Fisher's exact test. Mean and standard deviation were calculated for the variables. The data were analyzed by the statistical computer software SPSS, version 15.0 (SPSS Inc, Chicago). A p value of less than 0.05 was considered to be statistically significant.

RESULTS

The characteristics of the enrolled patients are presented in Table I. Age and gender did not significantly differ between the two groups. We identified only 1 (3.12%) patient of group A (i.e., grade I-II adenotonsillar hypertrophy) who presented uncomplicated PNE. In group B (i.e., grade III-IV adenotonsillar hypertrophy) 6 children (22.22%) were found to have uncomplicated PNE (p=0.040, Table I). There was no statistically significant difference between group A and group B in terms

Table I. C*haracteristics of the enrolled patients.*

Patients			
	Group A	**Group B**	***p***
Number patients	32	27	
F/M	14/18	11/16	0.051
Mean age (yrs)	6.31±1.21	6.52±1.33	0.52
Number patients with enuresis	1 (3.12%)	6 (22.22%)	0.040
Serum IgE levels (IU/ml)	215.25±18.81	211.93±38.41	0.43

Group A: AR patients with grade I-II adenotonsillar hypertrophy. Group B: AR patients with grade III-IV adenotonsillar hypertrophy.

of atopic sensitization (Table II) and serum total IgE levels (p=0.43, Table I).

DISCUSSION

PNE is a common disorder in the paediatric age group. Although several theories have been proposed to date to explain the underlying pathophysiology of this disorder, the exact etiological mechanism still remains unclear. It is likely that several conditions are implied in the onset of enuresis. Several previous studies reported children with allergy and/or allergy-related manifestations who had frequent PNE manifestations, suggesting an underlying etiological relationship between atopic disorders and nocturnal enuresis (14, 18-25). In line with these suggestions, a demographic study positively correlated the presence of PNE in association with atopic sensitization and allergic diseases, including asthma (25). Conversely, Siegel et al. (26) reported no relationship between atopic diseases and PNE. Our preliminary findings show that children with allergic rhinitis and grade III-IV adenotonsillar hypertrophy have a major incidence of PNE when compared to children with allergic rhinitis and a lower grade of adenotonsillar hypertrophy (i.e., I-II). According to other studies (27), we also detected no differences in serum IgE total levels between the two enrolled groups (i.e., A and B). Although IgE levels (when absent) may support the exclusion of an underlying allergic disease, it must be kept in mind that the total serum IgE values are not specific and/or sensitive diagnostic tests to diagnose atopic disorders (28). It has been previously postulated that atopy (directly and/or indirectly) may influence the likelihood of PNE in children. Environmental allergens are known to cause inflammation of the bladder, smooth muscle contraction and detrusor instability,

Table II. *Distribution of specific inhalant allergens*.

Inhalant allergens			
	Group A	**Group B**	***p***
House dust mite	8/32	5/27	0.36
Mixed grass	4/32	3/27	0.86
Parietaria	3/32	5/27	0.30
Birch	2/32	4/27	0.27
Olive tree	6/32	8/27	0.32
Alternaria	4/32	2/27	0.42
Cat epithelium	5/32	4/27	0.93
Dog epithelium	3/32	1/27	0.38

Group A: AR patients with grade I-II adenotonsillar hypertrophy. Group B: AR patients with grade III-IV adenotonsillar hypertrophy.

finally impairing functional bladder capacity (29, 30). Additionally, in children with inflammatory rhinitis, exposure of the upper respiratory airways to allergens may lead to nasal obstruction symptoms and progressive hyperplasia of the nasal lymphatic adenoid tissue (i.e., adenoids) due to the persistent cytokine release and the related chronic inflammation (31-34); additionally, also adenotonsillectomy is a cause of inflammation of the upper respiratory airways (35). Severe adenotonsillar hypertrophy is one of the most common risk factors for upper airway obstruction which can lead to OSAS-related sleep disorders and, potentially, to the risk of developing PNE (36-37). In this respect, a correlation between sleep disorders of various etiologies and PNE has already been assessed by previous studies (38-40). Interestingly, it has been shown that chronic airway obstruction overloads work of breathing, which increases negative intrathoracic pressure and cardiac load (with subsequent atrial expansion), leading to the release of higher levels of natriuretic peptides that may promote diuresis (41, 42). This mechanism is better depicted in a condition known as pseudotumor cerebri syndrome (PTCS), where OSAS (especially if a concomitant obesity is present) is responsible for an increasing of intra-abdominal pressure, which later increases intra-thoracic and cerebral venous pressure, leading to an impaired outflow of cerebrospinal fluid and intracranial hypertension (43-46).

The drawback of the present study is represented by the relatively small number of children studied with consequent possible selection bias in the final results. However, in light of the present and the previous findings (28-40), we suggest an underlying etiological relationship between severe respiratory complications due to allergic rhinitis (i.e., III-IV grade adenotonsillar hypertrophy, OSAS) and PNE in some children. Further studies will be needed to define the possible atopic predisposition in children affected by PNE. An adequate treatment of allergic diseases can downregulate the persistent allergic phlogosis in organ and/or system (e.g., including adenoid tissue and bladder) possibly promoting a reduction of manifestations related to PNE.

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PROBIOTICS AND ALLERGIC RESPIRATORY DISEASES

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Probiotics are able to restore microbiome and the normal intestinal permeability, improve the immunological function of gut barrier, and reduce the intestinal inflammatory response and the production of pro-inflammatory cytokine characteristics of local and systemic allergic inflammation. Clinical studies have demonstrated the efficacy of probiotics in the treatment of various clinical conditions such as atopic dermatitis and food allergies and in the primary prevention of atopy. Recent studies have shown that oral administration of certain probiotic exerts therapeutic effects in the treatment of allergic respiratory diseases such as asthma and rhinitis.

In recent years, allergic diseases have increased in all western countries and the cause of would appear to be related to both environmental changes and the western lifestyle. Allergic respiratory diseases are also characterized by bronchial hyper-responsiveness and lung inflammation. Additionally, airway inflammation and remodelling together likely explain the clinical manifestations of allergic respiratory diseases. Thus, spirometry measurements are necessary to evaluate lung function (1-4). There is a complex interaction between immunological, genetic and environmental factors that can influence the onset of allergic diseases particularly during prenatal life and the first years of life (5, 6). Many theories have been proposed to explain this phenomenon and the most followed one is still the "hygiene hypothesis", developed by Strachan in 1989 (7). According to this "hygiene hypothesis", the lack of exposure to microbial agents in early life modifies the responses of the immune system and increases the risk of developing allergic diseases (7, 8). Thus, children who live in countries with the best sanitary conditions have a greater predisposition to allergies, due to reduced antigenic stimulation environment. In these patients, the physiological switching of the immune system from Th1-Th2 would be compromised by a lack of development of immunological tolerance (7). Another theory, "microflora hypothesis", has suggested that the excessive use of antibiotics and changes in diet typical of industrialized countries, have altered the intestinal microbiome and the normal mechanisms of immunological tolerance (9). In support of these hypotheses several studies have shown that the gastrointestinal microflora is different in atopic subjects compared with non-atopic ones and in individuals who live in industrialized countries compared to those living in developing countries (10). Experimental data have also demonstrated that the endogenous bacterial flora play a significant role in modulating the development of the immune system (11). Probiotics are able to restore the normal microbiome and the intestinal permeability, improve the immunological function of gut barrier, and reduce the intestinal

Key words: probiotics, allergy, asthma, allergic rhinitis

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inflammatory response and the production of pro-inflammatory cytokine characteristics of local and systemic allergic inflammation (12, 13). Clinical studies have demonstrated the efficacy of probiotics in the treatment of various clinical conditions such as food allergies (14) and atopic dermatitis as well as in the primary prevention of atopy (15).

Recent studies have shown that oral administration of specific live probiotic strains may have therapeutic potential in the treatment of allergic respiratory diseases such as asthma and rhinitis (16, 17).

Probiotics and immune response in childhood

The activation of the innate immune system, caused by microbial stimulation, seems to have a key role in normal immune maturation. In a recent study the effectiveness of *Lactobacillus rhamnosus* in a murine model of "atopic march" was evaluated. Ha-Jung Kim et al., have shown that Th2, Th17, also producing IL-23 (18-21) and thymic stromal lymphopoietin (TSLP), are associated with the development of the "allergic march". *Lactobacillus rhamnosus* seems able to prevent the progression of atopy through the suppression of these cytokines by a mechanism that involves $CD4^+$ $CD25^+$ $Foxp3^+$ and Treg cells in mesenteric lymph nodes (18). Therefore, it is likely that the immunomodulatory effects of some probiotics in allergic diseases occur through their ability to modify dendritic cell (DC) function, in turn inducing the proliferation of a specific subpopulation of Tregs, such as Th3 and Th1, and suppress inflammatory response induced by allergens (22). The mechanism by which this modulation occurs is still unknown. However, it was shown that the cellular wall of Lactobacillus contains immunomodulator components, like some particles of the cellular surface and the peptidoglycan, that can have a very important role in the activation of immunocompetent cells (23). For example, it has been shown that lactobacilli can bind the lectin, which is an intercellular adhesion molecule for DC on TLR 2, 4 and 9 (23) which represent the first step in the stimulation of DC to promote the development of Treg cells that produce increased levels of IL-10, as in atopic dermatitis (24), which in turn is able to inhibit the proliferation of Th2 cells (23). A recent study show that atopy may be a risk factor for tyroid autoimmunity probably by a similar mechanism (25).

Probiotics in the prevention of allergic respiratory diseases

Primary prevention is the possibility of averting the onset of allergic diseases in high-risk children not yet sensitized. A systematic review and meta-analysis of randomized controlled trials was recently published, evaluating the association of probiotic supplementation during pregnancy or childhood with the onset of asthma and wheezing in childhood (26). Twenty studies with 4,866 children were analyzed, and the primary outcome was the clinical diagnosis of asthma. The results did not demonstrate a protective effect of the use of probiotics in the development of asthma or wheezing in infancy, thus the authors concluded that there is insufficient evidence to recommend the use of probiotics for primary prevention of asthma or wheezing. Similar results were also obtained from a recent meta-analysis conducted by Elzab and coll. (27). The results show that administration in the prenatal and/or early stages of life of probiotics reduces the risk of atopic sensitization and decreases the level of total IgE in children, but does not reduce the risk of asthma or wheezing in childhood.

Probiotics in the treatment of allergic respiratory diseases

The use of probiotics in the treatment of allergic diseases has been extensively analyzed by several studies mainly for the therapy of atopic dermatitis (AD), but also in food allergy, allergic rhinitis and asthma. For asthma, in our randomized DBPC study (28), we evaluated the effect of oral administration of *Lactobacillus reuteri* or placebo for 2 months on allergic airway inflammation assessed by exhaled nitric oxide (FeNO) in 50 children (6 to 14 years of age) suffering from mild persistent asthma and allergic to house dust mite. At the end of treatment, the values of FeNO showed a significant reduction ($P = 0.045$) only in the group treated with *L. reuteri*. The evaluation of cytokines in exhaled breath condensate showed increased levels of IL-10 ($P < 0.05$) and

a significant reduction of IL-2 ($P < 0.05$) only in the group treated with *L. reuteri*. No significant difference in FEV1 and asthma control assessed with the C-ACT was found between the 2 groups. Our data thus demonstrated that *L. reuteri* (108cfu) is effective in reducing bronchial inflammation in children with allergic bronchial asthma. With regard to rhinitis, Xiao et al. (29) evaluated the effects of oral administration of *Bifidobacterium longum* on Japanese cedar pollinosis (JCPsis) that affects nearly one in six Japanese during the pollen season. In a randomized, double-blind, placebo-controlled trial, 44 subjects with JCPsis received *Bifidobacterium longum* or placebo for 13 weeks during the pollen season. The results showed significant reduction of rhinorrhea, nasal obstruction, itching and sneezing in the *Bifidobacterium longum* group compared to the placebo group.

Interestingly, some genetic variants seem to influence the clinical response to probiotic administration in the atopic population (30); further supporting the evidence of a gene-environment interaction in childhood atopic disorders (30-32).

CONCLUSION

The effectiveness of the use of probiotics in the prevention and treatment of allergic respiratory diseases is still a matter of debate because the few studies carried out have produced conflicting results. Some studies have confirmed the anti-inflammatory and immunomodulatory activities of probiotics. The beneficial effects, however, appear to be species-specific, even though it still does not allow an unambiguous interpretation, and many other studies are certainly needed to draw definitive conclusions and also understand the involved mechanisms.

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SAFETY AND EFFICACY OF SUBLINGUAL SPECIFIC IMMUNOTHERAPY TO HOUSE DUST MITE USING A DIFFERENT DOSAGE: A PILOT STUDY

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The aim of this randomized open study was to evaluate the safety and efficacy of different dosages (2000 UI vs 4000 UI) of sublingual immunotherapy (SLIT) in patients with allergic diseases such as asthma associated to rhinitis and rhinoconjunctivitis sensitized to house dust mites. We enrolled 61 patients with a history of allergic asthma, and a positive skin prick test for Dermatophagoides (D.) pteronyssinus/farinae. Patients were randomly assigned to receiving SLIT at dosage of 2000 UI (Group A) or 4000 UI (Group B) maintenance dose. We evaluated: subjective symptoms using a Visual Analogic Scale (VAS), the amount of prescribed symptomatic drugs, bronchial reactivity to methacoline and side effects using a specific questionnaire. A significant improvement in symptoms, assessed by VAS, was observed with both SLIT doses with no significant differences between groups. The provocation dose of methacoline inducing a 20% fall of FEV1 significantly increased after 12 months only in the 4000 UI dose group. In conclusion, both monomeric allergoid dosages of SLIT (2000 UI and 4000 UI) are a safe and efficacy option to reduce symptoms in patients with allergic asthma caused by house dust mites. Moreover, both dosages are efficacious even to protect against airway reactivity but it seems that monomeric allergoid of SLIT at higher dosage (4000 UI) is better than at the lower dosage (2000 UI).

Asthma is one of the most common chronic diseases in children. It is estimated that the number of people in the world suffering from asthma can reach approximately 300 million (1, 2), representing a major cause of hospitalization, access to emergency care, school absence and poor quality of life, as demonstrated also in children with allergic rhinitis (3, 4). It is also clear that, among patients with asthma, there is a considerable range of phenotypes, significant heterogeneity concerning function of the airway epithelium, the degree of airway inflammation and airway hyper-responsiveness (5-7) and variable responsiveness to anti-inflammatory drugs, so that it would be more appropriate to refer to asthma as a syndrome, rather than a disease. In recent years allergen-specific sublingual immunotherapy (SLIT) has emerged as an effective and safe treatment option to prevent the progression

Key words: immunotherapy; sublingual; house dust mite; bronchial hyper-reactivity; safety; efficacy; dosage

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of allergic diseases (8, 9) influencing an increase of patient adherence to this therapy (10). SLIT has been proved effective at a large range of doses, from five to three hundred times the doses used for the traditional subcutaneous immunotherapy (11). To date, there is no final agreement on the most advantageous dosage, and it is noteworthy that, although uncommon, potentially fatal side effects have been reported also with SCIT (12). Therefore the aim of this study was to evaluate the safety and efficacy of different dosage (2000 UI *vs* 4000 UI) of SLIT in patients with asthma sensitized to house dust mites.

MATERIALS AND METHODS

In this randomized, open study we enrolled 61 patients, both children and young adults with a history of allergic asthma associated to rhinitis or rhinoconjunctivitis and a positive skin prick test for Dermatophagoides (D.) pteronyssinus/farinae Patients sensitized to seasonal allergens were excluded. All patients, in stable condition, were treated with a monomeric allergoid in tablets (Lais R, Lofarma SpA, Milan, Italy) containing both D. pteronyssinus and farinae (50% each). After three days of induction treatment with increasing doses of SLIT, patients were randomly assigned to *Group A* receiving a maintenance dose of 2000 allergic units (AU)/week and *Group B* receiving 4000 AU/week. All patients were guaranteed with symptomatic pharmacological treatment (antihistamines and nasal steroid). During the study period (1 year) we evaluated: 1) subjective symptoms using a Visual Analogic Scale (VAS) where a 0 score indicated no symptoms and 10 most severe symptoms; 2) amount of symptomatic drugs needed; 3) bronchial reactivity to methacoline; and 4) side effects, using a specific questionnaire. Safety and clinical assessment was performed every 4 months. Patients were asked to record in a diary any local or systemic side effects after administration of each dose. Bronchial challenge with methacholine was performed at baseline and after 12 months of treatment.

A total of 31 patients were included in the lower dose group and 30 in the higher dose group with no significant differences between groups for age, body mass index and pharmacological treatment. Asthma severity in most of the patients of both two groups resulted moderate persistent.

All characteristics of the patients are described in Table I.

RESULTS

A significant improvement of symptoms was observed in patients using a SLIT dose of 2000 UI (VAS score before and after treatment $P<0.001$), as well as using a SLIT dose of 4000 UI (VAS score before and after treatment $P<0.001$) (Fig. 1) with no significant differences between the two groups ($P=0.638$). Moreover, there was no significant difference between groups concerning the use of additional anti-allergic medications. The provocating dose (PD20) of methacoline producing a 20% fall of FEV1 significantly increased after 12 months in patients using a SLIT 4000 UI dose (PD20 at baseline and after treatment, $P<0.001$) but not in the patients using a 2000 UI dose (PD20 at baseline and after treatment P= ns) (Fig. 2). During the entire study period no systemic side effects were reported by the patients in either group.

DISCUSSION

The scientific debate aiming to define the best dosage in terms of safety and efficacy on the use of SLIT is still open, since this treatment seems to be effective within a wide range of doses. Our pilot prospective study performed on both adult and paediatrician patients sensitized to house dust mite showed that the two different dosages of SLIT (2000 UI *vs* 4000 UI) had similar safety and clinical efficacy in patients with allergic asthma, in terms of disappearance of symptoms and use of other anti-allergic medication. This is not in agreement with a recent meta-analysis in which patients with seasonal allergy treated with higher dosage of SLIT showed better results than patients treated with lower dosages (13).

Today it is known that among children with

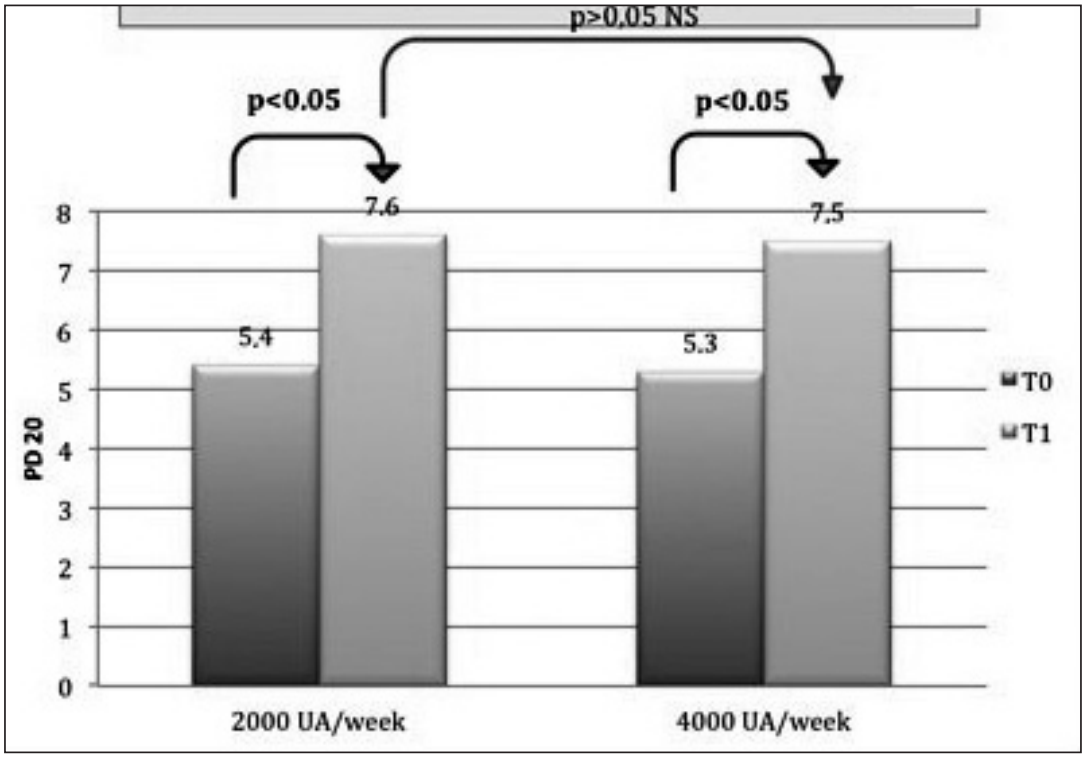

Fig. 1. *Changes in intra and inter-group V.A.S. at T0 and T1.*

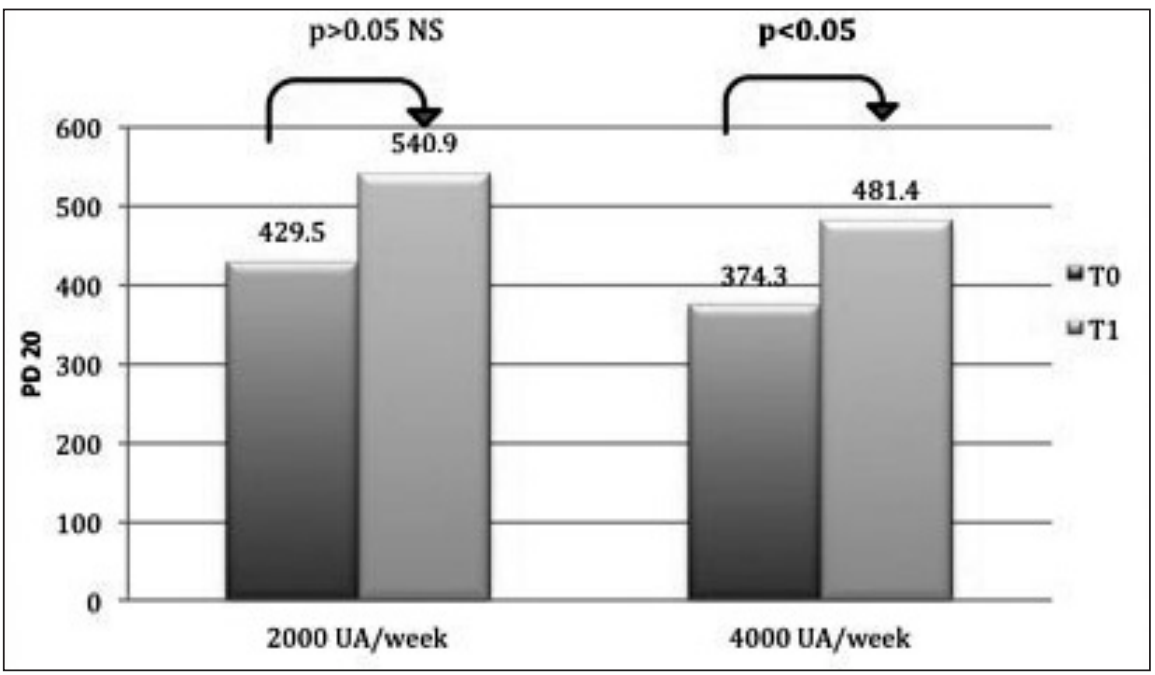

Fig. 2. *Variations intra- and inter-group PD20 at T0 and T*

asthma, there is a considerable range of phenotypes, significant heterogeneity concerning mucosal immunity, the degree of airway inflammation and airway hyper-responsiveness (14-17) and even serum markers (18-24) and this issue could play a key role on the open question regarding the "best dosage".

Effect of SLIT in bronchial hyperreactivity, airway inflammation and mucosal immunity was shown in our previous study (25). However, in the present study we found a significant improvement of bronchial hyperactivity only in children treated with higher dosage (4000 UI), while in the other group (2000 UI) we found no significant difference.

As progression of allergic rhinitis toward allergic asthma is a major objective to pursue with immunotherapy, the more significant effect of higher dosage on bronchial reactivity may justify the use of SLIT at a higher safe dosage.

In addition, the use of high dosage of the allergoid is encouraged by the lack of side effects and the optimal adherence to treatment, which is probably due to its low level of allergenicity (26-28).

In conclusion, we think that a dosage of 4000 UI or more of SLIT with a monomeric allergoid is as safe an option to reduce symptoms as a dosage of 2000 UI, and it seems to be more efficacious to protect against airway reactivity in patients with allergic asthma caused by house dust mites.

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Table I. *Characteristics of patients.*

	Group A : 2000 UA/week	**Group B: 4000 UA/week**
Number of patients	31	30
Sex (M/F)	21/10	18/11
Age (years)	26.5±12.8	28.2±18.9
Weight (kg)	65.6±17.8	63.8±16.9
Eight (cm)	163.1±16.4	164.8±14.9
Clinical condition		
Asthma	1	1
Rhinitis and asthma	25	23
Rhinoconjunctivitis and asthma	5	6
Severity		
Mild Persistent	26	25
Moderate Persistent	3	5

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RELATION OF BODY MASS INDEX WITH IgE LEVELS, ALLERGIC SENSITIZATION AND LUNG FUNCTION IN ASTHMATIC CHILDREN: OUR EXPERIENCE AND REVISION OF LITERATURE

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In recent decades, there has been an increase in the prevalence of asthma and obesity in pediatric age. In this regard several studies have provided controversial data to demonstrate the link between Body Mass Index (BMI) and asthma, both in adults and in children. In this prospective study we evaluated the relationship between body mass index value, total IgE immunoglobulin E levels, skin prick test (SPT) sensitization and lung function in children affected by asthma. According to the analysis of data on the comparison of normal-weight patients versus overweight/obese patients, there was no significant difference in the values of FEV1 (86%±12 vs 90% ± 19), FVC (81% ± 11 vs 88% ± 18), skin prick tests (22.72% vs 36.66%) and total IgE values (192.22±368.28 vs 503±914.04). We carried out a sub-analysis to study the difference between three groups of patients: normal weight, overweight and obese. Obese patients showed higher total IgE values than normal-weight patients with a statistically significant difference. Conversely, there was no significant difference between the normal weight group and the obese group in the respiratory function tests and the SPT. Moreover, we found a higher value of total IgE in female overweight/obese compared with normal weight, while there was no significant difference in relation to parameters of lung function and SPT. However, the same analysis in the male sample did not show any statistically significant difference. This study confirms the higher incidence of atopy in obese children, especially in female gender, but not a direct relationship with either allergens sensitization or abnormal lung function.

In recent decades, there has been an increase in prevalence of asthma and obesity in pediatric age (1, 2). However, while obesity can be attributed to lifestyle changes, the increase in asthma prevalence remains largely unexplained. Several studies provide controversial data on the relationship between obesity, atopy, allergens sensitization and lung function focusing on demonstrating the link between Body Mass Index (BMI) and asthma, both in adults and children (3). Recent studies suggest that systemic inflammation and inflammatory markers, as measured by C-reactive protein (CRP) levels, interleukins (IL-6 and IL-1) and High mobility group box 1 may be important in understanding this relationship since they are increased in obese subjects (4-9). The aim of our study was to evaluate the relationship between body mass index value, total IgE immunoglobulin E levels, skin prick test sensitization and lung function in children affected by asthma.

MATERIALS AND METHODS

We designed a case-control, prospective study, including children with a clinical history

Key words: asthma; obesity; children; allergic sensitization; lung function

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of asthma referred to our Institution for further examination. Diagnosis and classification of asthma was made according to GINA guidelines (10). Exclusion criteria were cystic fibrosis, sickle cell anemia, chronic lung disease of prematurity, interstitial lung disease and neurological disorders. The calculated needed sample was at least 50 subjects, so between September and July 2011 a total of 52 patients (39 males and 13 females; mean age 11±5 years) were consecutively enrolled. A detailed clinical history was taken and a complete physical examination was performed. All patients assumed only short-acting beta agonist or oral antihistamines on demand. After having collected the anthropometric data, the patients included in the study underwent the following investigations: Skin Prick Test (SPT) to inhalant and food (11); dosage of total IgE; and basal spirometry. The panel of employed allergens consisted of: house dust mites (*Dermatophagoides farinaee* and pteronyssinus), cat, dog, grasses mix, *Compositae* mix, *Parietaria officinalis*, birch, hazel, olive tree, *Alternaria tenuis*, Cladosporium, Aspergilli mix (Stallergenes, Milan, Italy). Dosage of total IgE was determined by Radio Immuno Sorbent Test (RIST); the cut-off value was of 90 UI/ml. Spirometry was performed by using a computer-assisted spirometer (Pulmolab 435-spiro 235, Morgan, England) and according to international guidelines. For each patient BMI was measured, calculated as weight (Kg)/height (m^2) ratio. Subsequently, patients were divided into two groups, according to BMI: normal weight children and overweight/obese children. Normal weight was defined by BMI between 18 and 24.99, overweight by BMI between 25 and 29.99, and finally children with BMI greater than 30 were considered obese. Continuous variables were presented as mean ± SD and were compared using Student's unpaired *t*-test or parametric analysis of variance (ANOVA) based on a test for normal distribution. Categorical variables were presented as count and percentage and were compared using *chi*-square test when appropriate (expected frequency> 5). Otherwise, Fisher's exact test was used. All tests were two sided and a P value less than 0.05 was considered statistically significant.

RESULTS

Among the patients studied (n= 52), 22 (42.3%) were normal weight, 22 (42.3%) were overweight and 8 were obese (15.4%). According to the analysis of data on the comparison of normal-weight patients versus overweight/obese patients, there was no significant difference in the values of FEV1 (86±12% *vs* 90±19%), FVC (81 11% *vs* 88±18%), SPT (22.72% *vs* 36.66%) and total IgE values (192.22±368.28 *vs* 503±914.04). Moreover, we performed a sub-analysis to study the difference between three groups of patients: normal weight, overweight and obese. In this case, obese patients showed higher total IgE values than normal-weight patients with a statistically significant difference (1442.68±1523.18 *vs* 192.22±368.28)**.** Conversely, there was no significant difference between the normal weight group and obese group according to respiratory function tests and the SPT (Table I). Comparing spirometric data, total IgE and results of SPT, we found lower values of FEV1 and FVC in males compared with females (86±15 *vs* 97±16; 82±15 *vs* 95±15, respectively; $p < 0.05$), although within normal range. Moreover, we reported a higher value of total IgE in female than in male gender (307.36±354.78 *vs* 1761.87±1552.23 $p < 0.05$). In particular, this increase was significantly higher in overweight/obese females than normal weight females, while there was no significant difference in relation to parameters of lung function and SPT (Table II). On the contrary, the same analysis in the males did not show any statistically significant difference (Table III).

DISCUSSION

To date several studies have provided controversial data about the relationship between obesity, atopy, allergen sensitization and lung function in asthmatic children, and consequently our data provide new information on this subject (12-17). On this issue also in our study we found a significant relation between obesity and atopy according to high IgE values but no relation between obesity or overweight and lung function according

Table I. *Anthropometric data, total IgE, spirometric values and SPT positivity in normal weight, overweight and obese patients.*

	Normal weight	Overweight patients	Obese patients	P value
N.	22	22	8	
M/F	16/6	19/3	4/4	
Age	10.18±2.30	8.68±2.76	12.25±2.37	ns*
Weight	41.45±8.26	51.15±11.75	81.52±14.41	<0,05*
Height	143.54±12.08	136.29±18.15	155.25±7.77	ns*
BMI	20.03±2.30	26.96±1.31	33.77±3.54	<0,05*
FEV1	86±12	89±16	93±24	ns*
FVC	81±11	86±17	93±22	ns*
Total IgE	192.22±368.28	189.78±224.72	1442.68±1523.18	<0,05*
Skin prick tests	5 (22%)	8 (36%)	3 (37%)	ns**

*ns=not significant; *= Anova Test; **= Fisher Test*

Table II. *Anthropometric data, total IgE, spirometric values and SPT positivity in normal weight and overweight/obese females.*

	Normal weight Female	Overweight/obese female	P value
N.	6	7	
Age	10.5±1.76	11.28±4.27	ns*
Weight	43.83±10.94	69.58±27.02	<0,05*
Height	147.16±12.23	147.57±23.92	ns*
BMI	19.96±2.29	30.88±4.98	<0,05*
FEV1	90±5	106±21	ns*
FVC	88±5	104±20	ns*
Total IgE	307.36±354.78	1761.87±1552.23	<0.05*
Skin prick tests positivity	3 (50%)	3 (42.85%)	ns**

*ns=not significant; *= Anova Test; **= Fisher Test*

Table III. *Anthropometric data, total IgE, spirometric values and SPT positivity in normal weight and overweight/obese males.*

	Normal weight male	Overweight/obese male	P value
N.	16	23	
Age	10.06±2.51	9.09±2.58	ns*
Weight	40.56±7.24	55.70±14.35	<0,05*
Height	142.18±12.14	139.34±16.41	ns*
BMI	20.05±2.38	27.95±3.00	<0,05*
FEV1	84±13	87±17	ns*
FVC	79±12	85±17	ns*
Total IgE	149.04±375.02	194.22±225.73	ns*
Skin prick tests positivity	2 (12.5%)	8 (34.78%)	ns**

*ns=not significant; *= Anova Test; **= Fisher Test*

to FEV1 and FVC. On the contrary, both Leung et al. (18) on Chinese children in 2009 and Ciprandi et al. in 2014 (19) demonstrated that obesity seems to be not associated with asthma oratopy. In support of those results, the authors cited the study conducted by Haldar et al. in 2008, which defined the "obese phenotype" as a cluster where airway inflammation is caused by a non-eosinophylic/ non-allergic mechanism (20, 21). Also studies analyzing allergen sensitization by SPT to evaluate the relationship between allergic asthma and obesity in children have given contrasting results such as atopy. In 2005 Vieira et al. observed that the presence of specific IgE was three times more elevated in obese women than in non-obese women, even if total IgE levels was similar between the two groups of women (22). Castro-Rodriguez et al. provided a significant correlation between BMI and SPT positive to common allergens or between BMI and asthma-like symptoms in adolescent females (23). In 2011 Cibella et al. concluded that overweight and obesity were independent risk factors for asthma and allergic sensitization (24). On the contrary Von Mutius et al. found that only the prevalence of asthma but not allergic sensitization defined as positive SPT to at least 10 allergens was significantly associated with BMI (25). Also our data seem to confirm this hypothesis since we found no significant difference of allergic sensitization between normal weight, overweight and obese children. All published studies highlight that only obese females seem to have a higher prevalence of atopy and allergen sensitization. Hancox et al. found that in the female group, besides an association between high BMI and asthma, there was a statistically significant association between high BMI and atopy (positive SPT and elevated serum IgE), but no correlation with the ratio FEV1/FVC (26). One year later, Kattan and coll. counteracted this hypothesis (27). Conversely, several studies demonstrated a significant relationship between BMI and asthma only in girls (28-30). Although it remains unclear, studies in literature seem to confirm the role of the

high levels of estrogens related to obesity in females. Authors showed that, alone or in combination with endogenous estrogens, environmental estrogens induced a release of mast cells and enhanced the release of other allergic mediators by an IgE-mediated mechanism (31). Moreover, Macsali demonstrated association between the use of oral contraceptive pill with an increased risk of asthma in overweight women (32). In the same year Romieu evaluated the role of estrogen-based hormone replacement therapy (HRT) in adult women and he demonstrated an increased risk of developing asthma in adult women using this treatment (33). In 2013, Abdul Wahab et al. dosed serum leptin and adiponectin to asthmatic children. They conclude that the variation of asthma risk in female and male genders would depend on adipokine plasmatic concentrations (34). Despite many doubts and few certainties, the correlation between asthma, obesity, atopy and female gender may be explained by the fact of the aromataseis being located in the adipose tissue; it is therefore reasonable to assume that obesity, characterized by increased levels of androgens, predisposes more to the aromatization of androstenedione to estrone and the testosterone to estrogensin peripheral tissues, determining an increase in female hormones. This latter induces mast cell degranulation with the release of allergic mediators through an IgE-mediated mechanism determining the atopic status. Moreover, estrogens may play a role in asthm aby modifying the inflammatory response toward a Th2 response(18, 28).

CONCLUSIONS

It is known that obesity prevalence is rising in the pediatric population, leading to an increase of the several obesity-related comorbidities and/or complications, including metabolic syndrome (35-39), endocrine deregulations (40-42), cardiovascular and neurological issues (43, 44), and allergy. This study confirms the evidence of a higher incidence of atopy in obese children, especially in female gender, but not a direct relation with both allergen sensitization and abnormal lung function.

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PROBIOTICS AND INFLAMMATORY BOWEL DISEASES

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Intestinal microbiota is composed by symbiotic innocuous bacteria and potential pathogens also called pathobionts. Even if the mechanism of action of intestinal bacteria remain still unknown, specific microbial species seem to have important role in the maintenance of immunological equilibrium in the gut through the direct interaction with immune cells. Some studies have found a dysregulated interaction between the intestinal bacteria, the gut barrier, and the intestinal associated immune system in Inflammatory Bowel Disease (IBD) patients and in the pathogenesis of these pathologies. In IBD patients some Butyrate producing bacteria, as Faecalibacterium Prausnitzii, are under represented and this could be related with their chronic inflammatory state.

Probiotics are known for their efficacy in the maintenance of intestinal homeostasis and microbiota. Several studies investigated their possible therapeutic use in IBD patients, both as single strain and as mixtures.

In this review we analyzed the most recent and representative studies regarding the use of probiotic bacteria in the treatment and prevention of IBD.

These studies, even if with some controversial results, show an overall promising trend. Probiotics could be useful for the prevention of IBD and maintenance of remission, supporting the classical pharmacological approach.

INTESTINAL MICROBIOMA IN HEALTHY SUBJECTS

Intestinal microbiota is composed by symbiotic innocuous bacteria and potential pathogens also called pathobionts (1). The human gut normally hosts roughly 10^{14} bacterial organisms of up to 1000 different species.

In total, the number of intestinal bacteria is approximately ten times the number of cells constituting the human body, with the collective bacterial genome, also referred to as the microbiome, containing 100-fold more genes than the entire human genome (2,3).

This bacterial community can add up to 1-2 kg (4) and is mainly composed by species within 4 bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (5,6).

Children's delivery mode and diet have a strong impact on early microbiota composition. Vaginally delivered children display a microbiota which reflects the maternal one and is usually composed by *Lactobacillus*, *Prevotella*, *Atopobium*, or *Sneathia spp*., while newborns delivered with caesarian section have a different colonization pattern, characterized especially by *Staphylococcus spp*. (7).

Intestinal microflora may modulate local and systemic immune responses, even if bacterial strains do not directly interact with immune cells because it is separated by the epithelial barrier that is composed by a physical, chemical and electrical component.

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In particular, aerobic species may have an effect on CX3CR1+ antigen presenting cells (APCs), promoting differentiation of Th17 cells through ATP production (8). These cells are involved in the development of oral tolerance may have a role in the protection against colitis (9).

$CX3CR1^+$ dendritic cells (DCs) may differentiate from monocyte precursors probably recruited in response to signals form microbiota. Another DCs subpopulation found in human mesenteric lymph nodes (MLN), the tolerogenic $CD103^+$ DCs, constitutively express indoleamine 2,3-dioxygenase (IDO), fundamental for their tolerogenic potential, are able to promote T cell differentiation via a mechanism dependent on retinoic acid and TGF-β and to drive T cells homing in the gut, even without a direct interaction with microbial strains (10). IL-10 signaling seems to be involved both in then maintenance of oral tolerance and in the prevention of intestinal inflammation activities, through the stimulation of T reg cells and the inhibition of T cell pro-inflammatory responses (11).

Even if the mechanism of action of intestinal bacteria remain still unknown, specific microbial species seem to have important role in the maintenance of immunological equilibrium in the gut through the direct interaction with immune cells. It has been demonstrated that *B. fragilis* may interact with DCs after Tight Junctions disruption or its polysaccharide A may cross the mucus layer for direct interaction with the epithelium and/or DCs (12).

Intestinal pathogens, almost by definition, have evolved mechanisms to subvert or avoid the secreted mucus barrier. They are virtually all motile, allowing them to move in mucus, and many bind mucin carbohydrates and produce enzymes that break down the mucin polymer network to facilitate penetration of mucus (13 - 17)

The protozoan parasite Entamoeba histolytica cleaves MUC2 in a manner likely to depolymerize the MUC2 complex, leading to loss of viscosity and disintegration of mucus (18).

In addition, many intestinal pathogens produce adhesins that allow them to dock onto the epithelial surface.

Intestinal bacteria are important for the maintenance of intestinal homeostasis also due to their ability to produce several metabolites, that can facilitate nutrients absorption, and short-chain fatty acids (SCFAs), organic acids produced by intestinal microbial fermentation of mainly undigested dietary carbohydrates. Among SCFAs, butyrate acid is mainly produced by Gram-positive anaerobic bacteria and seems to be the major energy source for colonocytes and to have an important role in the maintenance of colonic mucosal health. The most important groups of butyrate producers appear to be Faecalibacterium prausnitzii and Eubacterium rectale/Roseburia spp., which are key members of healthy adult gut microbiota and may undergo great variations at the phylum level together with Bacteroides uniformis species, expecially in IBD patients (19).

MICROFLORA IN IBD PATIENTS.

Inflammatory bowel diseases (IBD) include Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis and are characterized by alternating phases of clinical relapse and remission.

The 5–25% of IBD cases develop during childhood or adolescence, before 20 years of age (20). The early onset of the disease is often associated to a poorer prognosis. Crohn's disease onset is often characterized by small bowel disease associated with an extensive colon location, ulcers by widespread location at diagnosis and a high rate of disease extension. Complicated disease behaviour is more prevalent in IBD early onset (21).

Host intestinal microflora has a central role in sustaining the function and integrity of the epithelial barrier and its vascularization, in promoting the development and maintenance of gut-associated lymphoid tissue (GALT), and for the development of motility. Some studies have found that play a fundamental role in IBD a dysregulated interaction between the intestinal bacteria, the gut barrier, and the intestinal associated immune system (22).

Several lines of evidence strongly support the hypothesis of the involvement of intestinal microbiota in IBD pathogenesis. Crohn's disease (CD) and ulcerative colitis (UC) occur in the colon and distal ileum, which contain the highest intestinal

bacterial concentrations (5).

Fecal stream diversion prevents recurrence of CD in the neoterminal ileum, but reinfusion of luminal contents into bypassed colonic segments rapidly results in recurrent disease (23 - 24).

Similarly, ulcerative colitis patients who undergo ileal pouch-anastomosis surgery develop mucosal inflammation after bacterial colonization of the pouch (25).

Some nonabsorbable antibiotics may induce and maintain remission in IBD as shown in a recent meta-analysis by Khan et al (26).

Antibiotic treatment also appears to provide clinical benefits in patients with CD and inflammation of the ileal pouch (27).

Many studies on animal models support the role of gut microbiota in the development of IBD. In experimental animal models of IBD, genetically-engineered animals developed spontaneous colitis under standard laboratory conditions, but in germ-free environment colitis cannot be induced in animal models, thus indicating that bacterial exposure and colonization are essential for the development of colitis (28 - 30).

Additionally, it has been shown that animal models pretreated with antibiotics with chemically induced colitis do not develop intestinal inflammation (31).

The majority of genes associated with an increased risk for the development of IBD act to preserve the mucosal barrier and/or regulate the host immune system. The discovery of the NOD2/CARD15 gene is very important in understanding the linkage between genetic predisposition and IBD development, NOD2/CARD15 encodes a protein belonging to the family of pattern-recognition receptors responsible for microbial recognition, induction of antimicrobial genes, and control of the host adaptive immune response (32).

The genetic defects found in IBD CD patients might make these individuals particularly susceptible to infection by intracellular bacteria such as *Mycobacterium avium paratuberculosis*, *Listeria monocytogenes*, and adherent-invasive *Escherichia coli* (33).

Mutations in genes for toll-like receptors, as well as for the CARD4/NOD1 receptor, may also be associated with increased susceptibility for IBD (34 - 37).

Patients with IBD have increased intestinal permeability: intestinal mucus barrier is significantly altered in UC patients, particularly in terms of mucus composition and phospholipid concentration (38), altered function of defensins, antimicrobial peptides with bactericidal activities, might also be involved in IBD (39). This could promote bacterial translocation through the intestinal mucosa.

There are much evidence that intestinal microorganisms are required for the triggering and perpetuation of inflammation in IBD, It is unclear whether a single specific microorganism or a group of microbial agents with distinctive characteristics could be responsible, or if it is the altereted immune response to the dysbiosis of the commensal intestinal microbiota that plays the most major role.

Mycobacterium avium paratuberculosis was the major candidate as single etiologic agent in CD in the past. This bacterium cause granulomatous enterocolitis in cattle that closely resembles CD in humans (40), and a trial of antibiotic therapy with clarithromycin, rifabutin, and clofazimine has proved ineffective in disease activity in CD patients treatment (41).

Increased numbers of Escherichia coli (E. coli) strain LF82 was isolated from the ileum of CD patients. Several studies have shown that CD patients have a higher prevalence of adherent-invasive E. coli (AIEC) in ileal lesions, which indicates a specific association of AIEC with CD; AIEC proliferation has also been found in the colonic mucosa of UC patients (42-44). AIEC are able to adhere to and invade intestinal epithelial cells, they are capable of surviving and replicating within macrophages, and are known to induce the release of large amounts of pro-inflammatory cytokines, such as TNF-a, by the infected host cell (37).

Albeit microbial pathogens have been postulated to cause IBD, it is now generally accepted that commensal intestinal bacteria, play an important or even central role in the pathogenesis of inflammatory bowel disease, and provide the constant antigenic stimulation that continuously activates immune system to cause chronic intestinal injury (2 - 4).

The mechanisms proposed to drive pathogenic

immunologic responses to intestinal microbial antigens are four: microbial pathogens inducing intestinal inflammation, dysbiosis of commensal microbiota with a decreased of protective commensal bacterial species and the increase of aggressive commensal bacterial species, host genetic defects in controlling commensal microbiota, and the presence of defects in the regulation of the immune system. These mechanisms increase exposure of bacterial antigens to mucosal T cells or alter host immune responses to commensal bacteria (5, 45).

In a steady state the coexistence of commensal bacteria with microbes is due to the activation of a tollerogenic responses by epithelial cells, macrophages, dendritic cells, and T and B lymphocytes (46, 47).

In IBD, genetically predisposed individuals appear to lose the normal tolerance to commensal bacteria, leading to a chronically active inflammation process in which the microbiota provide constant stimulus for the host immune system, causing perpetuation of the disease (31).

Chronic inflammation might result from an immunologic misperception of indigenous flora as dangerous organisms or from the failure of normal regulatory activity on mucosal immune responsiveness to intestinal bacteria (48).

There are now increasing evidence that the relationship between intestinal microbes and the mucosa of susceptible individuals triggers a cascade of reactions that starts with the interaction of microbes with specific receptors on intestinal epithelial cells, dendritic cells, and other antigen-presenting cells, followed by the interaction of these activated cells with lymphocytes, resulting in their differentiation into Th1, Th17 and Th2 inflammatory responses with the production of a wide range of inflammatory mediators, and consequently leading to mucosal damage (49).

Bacterial recognition is dependent on transmembrane pattern recognition receptors of intestinal epithelial cells, including toll-like receptors (TLR) and the intracellular NOD-like receptor family (50, 51).

Ligation of these bacterial receptors stimulates central signaling cascades that include the nuclear factor-kappaB (NFκB) pathway, one of the key pathways in mucosal homeostasis that is shown to be elevated in the chronic inflammation tissue of the IBD (52).

Composition of gut microbiota in patients with IBD has been extensively studied over the last decade and With the development of culture-independent techniques such as metagenomic analysis, the disturbance of intestinal microbiota has been better described. Although methodologies and results may differ, some generalizations are possible (53).

Numerous studies revealed that fecal microbiota has a different composition in IBD patients compared to healthy controls. The decrease in biodiversity and depletion of the phyla Bacteroidetes and Firmicutes can be observed in feces/mucosa-associated microbiota among IBD patients. Furthermore, at the genus level, many potentially protective bacteria and normal anaerobic bacteria such as Bacteroides sp., Eubacterium sp. and Lactobacillus sp., are significantly decreased both in active UC and CD patients, and even in patients with inactive IBD. Similar findings were described for mucosa-associated microbiota, a bacterial population present on the mucosal surface that is in direct interaction with intestinal epithelial and immune system cells (54 - 57).

Moreover, differences were observed between active and non-active stages of the disease as well as between inflamed and non-inflamed regions of the intestine (58, 59).

Even though, the use of molecular techniques has greatly improved the detection rate, a significant numbers of bacteria can still be left undetected (60).

Many strains found in IBD do not belong to major phylogenetic groups represented in healthy individuals (61).

A distinction should be made between mucosal flora and fecal flora. The composition of these two domains is unique, which seems to be important in IBD (62).

Concentrations of mucosal bacteria are high in patients with bowel inflammation, whereas they are low in healthy controls. Bacterial invasion of mucosa was evident in up to 83% of biopsies from IBD patients, while no bacteria were detected in tissue samples from controls (60, 63).

Functional alterations are most evident in

adherent, invasive *Escherichia coli* that colonize the ileum of Crohn's disease patients (64).

Fluorescent in situ hybridization studies demonstrate dramatically increased mucosa-associated bacteria in active Crohn's disease, and to a lesser extent in ulcerative colitis (48). The fecal microbiota differs from the mucosa-associated microbiota (65), with the latter probably being more relevant for intestinal immunomodulation (56).

IBD AND PROBIOTICS

Evidences of the efficacy of probiotics in ulcerative colitis

Clinical studies on the efficacy of probiotics for the therapy of ulcerative colitis gave encouraging, albeit conflicting, results, especially for the maintenance of remission.

Several studies investigated the efficacy of VSL#3 a multispecies probiotic containing four strains of Lactobacilli (*L. casei, L. plantarum, L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), three strains of Bifidobacteria (*B. longum, B. breve*, and *B. infantis*) and one strain of Streptococcus (*S. salivarius* subsp. t*hermophilus*).

Low-dose balsalazide (2.25 g/d) plus 3 g/d VSL#3 was significantly superior to medium-dose balsalazide alone, and mesalazine in the 8-wk treatment of mild to moderate active ulcerative colitis in induction of remission (66).

An open-label study showed the efficacy and safety of VSL#3 3.6 × 109 CFU/d for induction of remission in 34 ambulatory patients with mild to moderate active UC. Remission (defined as UCDAI < or = 2) was achieved in 53% and the investigators reported no biochemical or clinical adverse events related to VSL#3 (67).

In a small open-label pilot study on 18 pediatric patients between the ages of 3-17 years with mild to moderate acute UC using VSL#3 for 8 wk remission (defined as SCCAI ≤ 3) was achieved in 56% with no change or worsening reported in 39% of patients (68).

VSL#3 risulted lightly efficacy in a dosage of 3.6×10^9 CFU (n = 65) versus placebo (n = 66) in achieving remission in UC patients on concomitant therapy with aminosalicylates and/or immunosuppressants (69). The primary end point of the study was to assess the effects of supplementation with VSL#3 in patients affected by relapsing ulcerative colitis (UC) who are already under treatment with 5-aminosalicylic acid (ASA) and/or immunosuppressants at stable doses. Remission was higher in the VSL#3 group than in the placebo group (47.7% vs 32.4%; P = 0.069). VSL#3 supplementation is safe and able to reduce UCDAI scores this patients. Moreover, VSL#3 improves rectal bleeding and seems to reinduce remission in relapsing UC patients after 8 weeks of treatment, although these parameters do not reach statistical significance.

The efficacy of VSL#3 applied twice daily for 12 weeks in a dosage of 3.6×10^9 CFU (n = 77) versus placebo (n = 70) for induction of remission of mild to moderate UC in adult patients was highlighted in a randomized, multicenter, double-blind, controlled trial (70).

The primary end point was a 50% decrease in the Ulcerative Colitis Disease Activity Index (UCDAI) at 6 weeks. The secondary end points included remission by 12 weeks and reduction in total individual UCDAI parameters from baseline at 12 weeks. Intention-to-treat analysis was performed. At week 6, the percentage of patients with an improvement in UCDAI score that was greater than 50% was significantly higher in the group given VSL#3 (25; 32.5%) than the group given placebo (7; 10%) (P = .001). At week 12, there were 33 patients given VSL#3 (42.9%) who achieved remission, compared with 11 patients given placebo (15.7%) (P < .001). Furthermore, significantly more patients given VSL#3 (40; 51.9%) achieved a decrease in their UCDAI that was greater than 3 points, compared with those given placebo (13; 18.6%) (P < .001). The VSL#3 group had significantly greater decreases in UCDAI scores and individual symptoms at weeks 6 and 12, compared with the placebo group.

A 1-year prospective, placebo-controlled, double-blind pediatric study assess the efficacy of VSL#3 on the induction and maintenance of remission in children with active UC (71)

A total of 29 consecutive patients (mean age: 9.8 years; range: 1.7-16.1 years) with newly diagnosed

UC were randomized to receive either a weight-based dose of VSL#3 (n = 14) or placebo (n = 15) in conjunction with concomitant steroid induction and mesalamine maintenance treatment. All 29 patients responded to the induction therapy. Remission was achieved in 92.8% children treated with VSL#3 and standard therapy compared to only 36.4% treated with placebo and standard therapy ($P < 0.001$). Moreover, only 21.4% patients treated with VSL#3 relapsed within 1 year of follow-up compared to 73.3% patients from the placebo group ($P = 0.014$). At 6 mo, 12 mo, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL#3 group than in the placebo group ($P < 0.05$). There were no biochemical or clinical adverse events related to VSL#3. This study demonstrated the efficacy of VSL#3 both in the induction and maintenance of remission in pediatric UC patients.

In a single-center, randomized, double-dummy study examined whether the addition of a non-pathogenic strain of *E. coli* Nissle 1917 to standard medical therapy increased the chance of remission of active ulcerative colitis and whether this probiotic strain was as effective as mesalazine in preventing relapse. Of a total of 116 patients, 59 were randomized to the mesalazine group and 57 to the E. coli group (72).

After remission, patients were maintained on either mesalazine or E. coli, and followed-up for 1 year. The investigators found no significant differences between the mesalazine and E. coli groups in percentage of patients that achieved remission, mean time to remission, percentage of patients who relapsed, and mean duration of remission. Although the addition of E. coli to standard therapy did not increase the induction rate of remission, the results suggested that treatment with this probiotic might have an equivalent effect to mesalazine in maintaining remission of ulcerative colitis (73).

Other studies was performed using BIO-THREE (containing *Streptococcus faecalis, Clostridium butyricum,* and *Bacillus mesentericus*), Bifidobacteria-fermented milk (BFM) (containing *Bifidobacterium breve* strain Yakult, *B. bifidum*, and *Lactobacillus acidophilus*) supplementation as a dietary adjunct in treating active ulcerative colitis with encouraging results (74, 75).

To assess in children with active distal UC the effectiveness of Lactobacillus (L) reuteri ATCC 55730 enema on inflammation and cytokine expression of rectal mucosa, a total of 40 patients (median age: 7.2 years range 6-18) with mild to moderate UC were enrolled in a prospective, randomised, placebo-controlled study (76). They received an enema solution containing 10^{10} CFU of *L. reuteri* ATCC 55730 or placebo for 8 weeks, in addition to oral mesalazine. Clinical endoscopic and histological scores as well as rectal mucosal expression levels of IL-10, IL-1β, TNFα and IL-8 were evaluated at the beginning and at the end of the trial. Thirty-one patients accomplished the trial (17 males, median age 13 year, range 7-18). Mayo score (including clinical and endoscopic features) decreased significantly in the *L. reuteri* group (3.2 ± 1.3 vs. 8.6 ± 0.8, $P < 0.01$) compared with placebo (7.1 ± 1.1 vs. 8.7 ± 0.7, NS); furthermore, histological score significantly decrease only in the *L. reuteri* group (0.6 ± 0.5 vs. 4.5 ± 0.6, $P < 0.01$) (placebo: 2.9 ± 0.8 vs. 4.6 ± 0.6, NS). At the post-trial evaluation of cytokine mucosal expression levels, IL-10 significantly increased ($P < 0.01$) whereas IL-1β, TNFα and IL-8 significantly decreased ($P < 0.01$) only in the *L. reuteri* group. In children with active distal ulcerative colitis, rectal infusion of *L. reuteri* is effective in improving mucosal inflammation and changing mucosal expression levels of some cytokines involved in the mechanisms of inflammatory bowel disease. concerning the induction of remission in ulcerative colitis by probiotics several reviews and meta-analyses have been performed over recent years.

In a Cochrane Collaboration review from 2007 the authors assessed the efficacy of probiotics compared to placebo or standard medical treatment with 5-aminosalicylates, sulfasalazine, or corticosteroids (77).

The authors concluded that combining conventional therapy with probiotics did not improve overall remission rates in patients with mild to moderate UC. The negativistic opinion shared in this early review can be at least partially attributed to the low number of high quality studies published at the time.

In contrast, a meta-analysis performed by Zigra et al (78) showed a significant benefit of probiotic

use for UC remission induction with pooled relative risk 2.27 (95%CI: 1.00-5.14, P = 0.049).

In a more recent review the only probiotic with several published randomized controlled studies for induction of remission in adult patients with UC was VSL#3 (79). The calculated pooled RR for VSL#3 was 1.69 (95%CI: 1.17-2.43), indicating a significant benefit of VSL#3 over control in inducing remission in active UC.

In the 3rd Yale Workshop recommendations for probiotic use of 2011, both VSL#3 and *Escherichia coli* Nissle 1017 were rated B, meaning that recommendation of their use for induction of remission in UC is based on positive controlled studies, but with the presence of some negative studies that did not support the primary outcome (80).

In conclusion, the results of several clinical studies suggest that the addition of specific probiotics to conventional therapy in active UC may be beneficial. The strongest evidence exists for multispecies preparation VSL#3, with several studies both in adults and children supporting its efficacy.

There have been several published studies in which efficacy of the probiotic strain of *Escherichia coli* Nissle 1917 was compared to either placebo or standard therapy for maintenance therapy in UC. Three study showed the efficacy of *Escherichia coli* Nissle 1917 in the maintenance of remission in UC compared to mesalazine. Authors concluded that *E. coli* Nissle 1917 showed the same equivalent efficacy and safety as mesalazine in maintaining remission in patients with ulcerative colitis (72, 81, 82).

A probiotic strain of *Lactobacillus rhamnosus GG* for maintenance therapy in UC where treatment with *Lactobacillus GG* alone or in combination seemed to be more effective than standard treatment with mesalazine in prolonging relapse-free time (P < 0.05) (83).

A non-controlled trial using multispecies preparation VSL#3 in 20 UC patients in remission, intolerant, or allergic to 5-aminosalicylates for 12 mo was performed and reported that 15 out of 20 patients remained in remission during the study, 4 relapsed, and one was lost to follow-up. They suggested that VSL#3 might be useful in maintaining remission in UC patients intolerant to standard therapy (84).

In the previously mentioned pediatric study by Miele et al (71), the investigators observed that only 21.4% of patients treated with VSL#3 (compared to 73.3% patients from the placebo group) relapsed within 1 year of follow-up (P = 0.014). They also found significantly lower endoscopic and histological scores in the VSL#3 group than in the placebo group (P < 0.05). The results of this study confirmed the efficacy of VSL#3 in the maintenance of remission in pediatric UC patients.

Other studies have shown the efficacy of some probiotic versus placebo.

BIFICO oral capsules of live combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus* (1.26 g/d) for 8 wk (85), *Lactobacillus salivarius subspecies salivarius UCC118*, *Bifidobacterium infantis 35624* (1×10^9 CFU/d) (86) and Probio-Tec-AB-25 (containing the two probiotic strains *Lactobacillus acidophilus La-5* and *Bifidobacterium animalis subspecies lactis BB-12*) (87) showed the efficacy of *Escherichia coli* Nissle 1917 in the maintenance of remission in UC.

A meta-analysis by Jonkers et al. (88) revealed that pooled relative risk for *E.coli* Nissle compared to mesalazine was 1.08 (95% CI 0.86-1.37), indicating that this strain of E. coli was not inferior to mesalazine in preventing relapses.

Recently a meta-analysis take in consideration five studies with 441 patients were identified. The pooled remission rate was 49.4% (95% CI, 42.7-56.1). Only 3 low risk of bias studies with 319 patients met the inclusion criteria for further analysis. A total of 162 patients received 3.6 × 10 CFU/d VSL#3, and 157 patients received placebo. A total of 95% of patients received concomitant therapies with 5-ASA and/or immunomodulators. The Ulcerative Colitis Disease Activity Index was used to define response and remission. A >50% decrease in the Ulcerative Colitis Disease Activity Index was achieved in 44.6% of the VSL#3-treated patients versus 25.1% of the patients given placebo (P = 0008; OR, 2.793; 95% CI, 1.375-5.676; number needed to treat = 4-5). The response rate was 53.4% in VSL#3-treated patients versus 29.3% in patients given placebo (P < 0001; OR, 3.03; 95% CI, 1.89-4.83; number needed to treat = 3-4). The remission

rate was 43.8% in VSL#3-treated patients versus 24.8% in patients given placebo (P = 0007; OR, 2.4; 95% CI, 1.48-3.88; number needed to treat = 4-5). No serious side effects were reported. The conclusions was thatVSL#3, when added to conventional therapy at a daily dose of 3.6 × 10 CFU/d, is safe and more effective than conventional therapy alone in achieving higher response and remission rates in mild to moderately active ulcerative colitis (89).

The American Recommendations for probiotic use from 2011 state very strong "A" recommendations for the use of the two specific probiotics *Escherichia coli* Nissle 1917 and multispecies mixture VSL#3 for the maintenance of remission in UC (80).

In conclusion, specific probiotics such as *Escherichia coli* Nissle 1917 and multispecies mixture VSL#3 are probably as efficient as standard maintenance therapy with mesalazine, and can therefore be used instead of mesalazine in patients intolerant or allergic to 5-amino-salicylates, or as adjunctive therapy to standard therapy, to potentially increase the duration of remission.

In some patients with UC in whom the disease does not respond to medical therapy or who develop dysplasia or cancer, proctocolectomy with the construction of ileal pouch-anal anastomosis (IPAA) is required. Inflammation of this ileal reservoir (pouch), referred to as pouchitis, develops in between 15% and 50% of such patients.

Evidences of the efficacy of probiotics in pouchitis

Although the exact etiology of pouchitis is not clear, host genetic factors, fecal stasis, mucosal ischemia, and bacterial dysbiosis in the pouch seem to be involved.

Most patients develop pouchitis in the first year after the procedure. Antibiotic therapy is generally successful; however, discontinuation of antibiotics is often followed by recurrence of the disease. Treatment and prevention of pouchitis with probiotics has thus been studied extensively, and only a few studies addressing the use of probiotics for the treatment of active pouchitis were published.

A double-blind placebo-controlled trial investigate the efficacy of *Lactobacillus rhamnosus GG* supplementation as primary therapy for ileal pouch inflammation. The authors concluded that although probiotic supplementation with *Lactobacillus GG* changed pouch microbiota, it was clinically ineffective as primary therapy for active pouchitis (90).

In an open-label study 51 UC patients with IPAA, 6 UC patients with ileorectal anastomosis without pouch, and 10 patients with IPPA because of familial adenomatous polyposis was treated with a fermented milk product culture, containing probiotic strains *Lactobacillus acidophilus La-5* and *Bifidobacterium animalis* subsp. *lactis* BB-12, in a dosage of 5×10^{10} CFU/d for 4 wk (91).

Before, during, and after intervention, symptom assessment and endoscopic evaluation was performed. Symptoms, such as involuntary defecation, leakage, abdominal cramps, fecal number and consistency, mucus, and urge to evacuate stools were significantly decreased during intervention in the UC/IPAA group. The median endoscopic score of inflammation also significantly decreased.

The efficacy of high-dose VSL#3 in the treatment of mild active pouchitis was evaluated in an open-label non-controlled study (92). Twenty-three patients with mild pouchitis were treated with VSL#3 (3.6×10^9 CFU/d) for four weeks. Symptomatic, endoscopic, and histologic evaluations were undertaken before and after treatment according to PDAI. Remission was defined as a combination of a PDAI clinical score of ≤ 2, an endoscopic score of ≤ 1, and a total PDAI score of ≤ 4. Patients in remission after initial treatment were treated with a maintenance dose of VSL#3 ($1.8\ 10^9$ CFU /d) for an additional six months. Sixteen out of 23 patients (69%) were in remission after treatment. The median total PDAI scores before and after therapy were 10 (range, 9-12) and 4 (range, 2-11), respectively ($P < 0.01$). The median Inflammatory Bowel Disease Questionnaire score also significantly improved ($P < 0.001$). All 16 patients who went into remission maintained remission during maintenance treatment. The authors conclude that high doses of the probiotic VSL#3 were effective in the treatment of mild pouchitis.

As pouchitis is a recurrent state, many studies evaluated the potential of probiotics in preventing

exacerbations. The most profoundly studied probiotic for this indication was multispecies preparation VSL#3.

Two randomized, double-blind, placebo-controlled trial, evaluated the efficacy of VSL#3 in the remission maintenance of chronic pouchitis compared with placebo (93, 94).

The authors concluded that oral administration of VSL#3 9 $\times 10^{11}$ CFU/d is effective in preventing flare-ups of chronic pouchitis.

In another study the researchers evaluated the effectiveness of a single daily high dose probiotic preparation of VSL#3 (9×10^{11} CFU) in maintaining antibiotic-induced remission. All patients included in this study had pouchitis at least twice in the previous year or required treatment with continuous antibiotics. After remission was induced within four weeks of combined metronidazole and ciprofloxacin therapy, the patients were randomized to receive either VSL#3 (9×10^{11} CFU) (n = 20) or placebo (n =16) once daily for one year or until relapse. Symptomatic, endoscopic, and histological evaluations were made before and 2 and 12 mo after randomization or at the time of relapse. Remission was maintained for one year in 17 patients (85%) on VSL#3, but in only one (6%) on placebo ($P < 0.0001$). The IBDQ score remained high in the VSL#3 group but deteriorated in the placebo group ($P = 0.0005$). Therefore, the authors concluded that the once daily high dose probiotic VSL#3 was effective in maintaining antibiotic introduced remission in patients with recurrent or refractory pouchitis (95).

In an open-label trial 31 patients at different periods after surgery without signs or symptoms of pouchitis were randomized to VSL#3 9×10^{11} CFU/d or no treatment for 12 mo (96).

Pouchitis activity was evaluated by PDAI, with different immunologic parameters being studied in peripheral-blood mononuclear cells and mucosal biopsies to reveal the mechanisms of probiotic action. During the study period, none of the patients from the probiotic group and only one from the placebo group developed active pouchitis. Because of the extremely low relapse-rate, even in the non-treated group, it was impossible to derive any firm conclusions regarding the efficacy of probiotic treatment from this study. However, a significant reduction in PDAI score was observed in VSL#3 treated patients.

In the Cochrane Collaboration review published in 2010, different modalities for the treatment and prevention of pouchitis after ileal pouch-anal anastomosis for UC, including different antibiotics, probiotics, glutamine, butyrate, and budesonide, were meta-analyzed and reviewed. They concluded that *Lactobacillus GG* was not superior in effectiveness compared to placebo for the treatment of acute pouchitis, while VSL#3 was more effective than placebo in the maintenance therapy of chronic pouchitis (97% vs 3%, $P < 0.0001$) (97).

Similarly, in a strain specific meta-analysis performed by Jonkers et al. (88), the authors calculated the pooled relative risk for prevention of relapses of pouchitis for VSL#3 compared to placebo as 0.17 (95%CI: 0.09-0.33).

In the American Recommendations for probiotic use from 2011, the multispecies probiotic preparation VSL#3 was granted the A level recommendation for the primary prevention and maintenance of remission of pouchitis after IPAA (80).

Furthermore, they suggested that there was some evidence (C level) supporting its use even for the therapy of active pouchitis.

Finally, clinical guidelines for the management of pouchitis from 2009 suggest the use of VSL#3 in patients with recurrence of pouchitis following antibiotic treatment or having several recurrences despite antibiotic therapy. However, they do not suggest probiotics for the treatment of acute pouchitis (98).

Evidences of the efficacy of probiotics in Crohn's disease

Clinical studies investigating the treatment of active Crohn's disease with probiotics were scarce and with a low number of patients.

A very small open-label pilot study reported of four children with mildly to moderately active Crohn's disease who were treated with entero-coated tablets containing *Lactobacillus rhamnosus GG* (10^{10} CFU) twice daily for 6 mo. A significant improvement in clinical activity was observed 1 wk after starting *Lactobacillus GG*. Median PCDAI scores at 4 wk

were 73% lower than baseline. Intestinal permeability improved in an almost parallel fashion. The authors concluded that the findings of this pilot study showed that *Lactobacillus GG* might improve clinical status and gut barrier function in children with mildly to moderately active Crohn's disease (99).

A small randomized, placebo-controlled trial performed the efficacy of *Lactobacillus rhamnosus GG* in the induction or maintenance of medically-induced remission. Eleven patients with moderate to active Crohn's disease were enrolled to receive either *Lactobacillus GG* (2×10^9 CFU/d) or placebo for six months. In all patients, a tapering steroid regimen was applied for the induction of remission, and all received antibiotics the week before probiotic/placebo intervention was initiated. The primary end point was sustained remission; defined as freedom from relapse after 6 mo. Only 5 patients finished the study, with 2 patients in each group in sustained remission. The median time to relapse was 16 +/- 4 wk in the probiotic and 12 +/- 4.3 wk in the placebo group (P = 0.5). In contrast with the results of Gupta et al (99), this study did not demonstrate any benefit of *Lactobacillus GG* in inducing or maintaining medically-induced remission in CD (100).

The Cochrane Collaboration review of 2008 (100) concluded that there was insufficient evidence to make any conclusions about the efficacy of probiotics in inducing remission in CD because of a lack of well-designed clinical studies.

Two studies using synbiotics that were not included in this review revealed very promising results (101, 102).

In a open-label uncontrolled trial ten active CD patients who had failed to achieve remission via an initial therapeutic regimen of aminosalicylates and prednisolone were given synbiotic therapy consisting of two probiotic preparations that both contained *Bifidobacterium breve* 3×10^{10} CFU, *Lactobacillus casei* 3×10^{10} CFU, and *Bifidobacterium longum* 1.5×10^{10} CFU, as well as a prebiotic comprised of 3.3-9.9 g of psyllium (Plantago ovata) (101). Patients were free to adjust their intake of probiotics or prebiotics throughout the trial. For the assessment of disease activity, Crohn's disease activity index (CDAI), International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score, and blood sample variables were evaluated and compared before and after the trial. The duration of the trial was 13.0 +/- 4.5 mo. Of the ten included patients, six had a complete response, one had a partial response, and three were non-responders. Two patients discontinued treatment and four decreased their corticosteroid therapy. Both CDAI and IOIBD scores were significantly reduced after therapy (255-136, P = 0.009; 3.5-2.1, P = 0.03, respectively), however the laboratory markers of inflammation did not change. With the exception of some abdominal bloating disappearing with discontinuation of psyllium ingestion, there were no adverse events. The authors concluded that a combination of high-dose probiotics and prebiotics could be safely and effectively used as a co-therapy for the treatment of active CD.

In a randomized, double-blind, placebo-controlled trial was include 35 patients with active CD using a synbiotic therapy comprised of probiotic *Bifidobacterium longum* 4×10^{11} CFU and prebiotic Synergy 1 (oligofructose and inulin) 12g daily (102). Patients were requested to continue on stable doses of conventional medication they were receiving at initiation of the trial. The patients' clinical status was scored by CDAI and endoscopies with biopsies were performed at the start, and at 3 and 6 mo of therapeutic intervention. Six patients from the synbiotic group and 5 from the placebo group were lost from follow-up. Upon comparing pre and post-treatment CDAI, there was a significant clinical improvement in the synbiotic group (start 219 ± 74.6, finish 147 ± 74; P = 0.020) but not in the placebo group (start 249 ± 79.4, finish 233 ± 155; P = 0.81). Similarly, there was a significant improvement in mean histological scores in the synbiotic group (start 6 ± 5, finish 3 ± 4; P = 0.018) but not in the placebo group (start 6 ± 5, finish 5 ± 6; P = 0.75). A significant reduction of pro-inflammatory TNF-α and an increase of mucosal Bifidobacteria was also observed in the synbiotic group.

Only a few high-quality studies have been performed to assess the efficacy of probiotics for the maintenance of remission achieved with standard medical therapy or surgical resection in Crohn's disease. Currently, the use of probiotics for the

maintenance of remission in Crohn's disease is not recommended. In a trial by Guslandi et al (103), 32 patients with Crohn's disease in clinical remission (CDAI < 150) were randomized to treatment with either mesalamine 1 g three times daily or mesalamine 1 g two times daily plus probiotic yeast *Saccharomyces boulardii* 1 g daily for six months. Clinical relapses were observed in 37.5% of patients receiving mesalamine alone but in only 6.25% of patients combining mesalamine with the probiotic (P = 0.04). The authors hence concluded that *Saccharomyces boulardii* might be useful in the maintenance treatment of Crohn's disease.

FUTURE DIRECTIONS IN INFLAMMATORY BOWEL DISEASE MANAGEMENT

It is now established that some of the bacteria that dominate the intestinal mucosal and luminal ecosystems of healthy subjects are under-represented in IBD: *Butyricicoccus pullicaecorum* and *Faecalibacterium prausnitzii*.

A study shows that subjects with inflammatory bowel disease (IBD) have lower faecal counts of *Butyricicoccus pullicaecorum*, that this bacterium attenuates trinitrobenzenesulfonic acid-induced colitis in rats and that the supernatant of its culture strengthens the epithelial barrier of Caco-2 cells (104). More generally, the question is whether those microbes might be used as diagnostic markers or as treatment options.

Candidates include Faecalibacterium prausnitzii, Butyricicoccus, Roseburia and the so called 'enterotypes', that is, complex ecosystems with co-occurring bacteria.

Several species among the bacteria under-represented in IBD have protecting properties against some experimental models of colitis, and therefore the question is whether they could be used as treatments. Interestingly, many of them are butyrate-producing bacteria. Butyrate itself sometimes has protective value in experimental colitis, but this is not always the case (105, 106).

Learning the microbial ecology of these species and how to maintain or increase butyrate levels or butyrate-producing bacteria thus seems a rational and promising step to improve treatments of IBD.

Intestinal and colonic bacteria gain energy from dietary substrates that escape digestion and host secretions including mucins. Owing to the proximal fermentation of some alimentary substrates, the respective part of host-derived substrates is higher in the distal colon.

Butyrate can be synthesised by two metabolic pathways: butyrate kinase and butyryl-CoA:acetate CoA-transferase, the latter being the dominant one in the human colonic ecosystem (107).

Certain dietary substrates lead to a higher intracolonic production of butyrate than others and are thus referred to as 'butyrogenic'. They include inulin, fructo-oligosaccharides, resistant starch and ispaghula.

They may stimulate butyrate production directly by certain groups of fibrolytic and amylolytic bacteria, or indirectly, for example, by cross-feeding some butyrate producers with lactate which is generated by lactobacilli and bifidobacteria during fermentation.

Noticeably, several of the butyrogenic substrates are also 'bifidogenic' and 'prebiotic' (in other words, they increase colonic concentrations of bifidobacteria). *Eubacterium hallii*, *Anaerostipes caccae* and *Escherichia coli* are some of the endogenous butyrate-producing bacteria that use lactate as a substrate thus they can be cross-fed by lactic acid bacteria which tend to co-occur with them. This may explain the pharmabiotic properties of some bifidobacteria and lactobacilli (108, 109). By contrast, *F. prausnitzii* and *Roseburia spp*. (the major endogenous bacteria that produce butyrate) are unable to use lactate (110)

The two phylogroups of *F. prausnitzii* can ferment several sugars, uronic acids and the host-derived N-acetylglucosamine. Despite being extremely oxygen sensitive, they can survive close to the gut mucosa owing to an extracellular electron shuttle of flavins and thiols (which are present in the healthy human gut but might lack in IBD) (111, 112). Butyrate itself may be butyrogenic; for example, Li et al (113) showed that experimental infusion of butyrate in the rumen had a stimulating effect on butyrate-producing bacteria. Bifidobacteria and lactobacilli

are easy to grow, but the therapeutic efficacy of the few strains tested in IBD seems limited despite some hope with the VSL 3 product (88)

Recently the group of Venessa Eeckhaut (114) showed that *Butyricicoccus pullicaecorum* easily grows under anaerobic conditions in M2GSC medium reaching 10^8CFU/mL after 24 h incubation. The same group further evaluated the survival and viability of *B. pullicaecorum* in hydroxypropylmethylcellulose (HPMC) capsules after they were anaerobically lyophilised in horse serum supplemented with 7.5% trehalose and 1 mg/mL cysteine HCl and only observed a reduction of 1 log after 7 months. Now, after 15 months of storage at 4°C, still 10^7CFU, *B. pullicaecorum* are counted per capsule which were initially filled with 10^8CFU, suggesting a potentially acceptable shelf-life. In addition, the formulation can still be optimised by testing appropriate additives (such as different lyoprotectants), and if the capsules are filled with higher numbers (eg, 10^9 CFU or more) adequate bacterial numbers can be provided to patients. In conclusion the data provided show that the strain *B. pullicaecorum* can be produced in a relatively stable freeze-dried form in HPMC capsules and exerts an equivalent effect compared with standard therapy in a TNBS model based on a significant reduction of the colon to body weight ratio (=colon index) and colon weight to colon length ratio. The next step will be the administration of HPMC capsules with freeze-dried *B. pullicaecorum* to healthy persons to evaluate intestinal colonization and the effect on the intestinal microbiota composition, followed by clinical trials aiming to prolong the remission period in inflammatory bowel disease patients (114).

There is a growing interest on *F. prausnitzii* in this field, but this anaerobic bacterium is difficult to maintain in "in vitro" and it has not been possible to evaluate its therapeutic effects in humans until now. By now, active products secreted in its supernatant are currently being investigated.

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SERUM YKL-40 IN CHILDREN WITH ASTHMA

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Asthma is one of the most common chronic diseases in children. To date the diagnosis of asthma is mainly clinical, based on the clinical history, a careful physical examination and lung function tests. However, symptoms are often not specific and lung function tests are not very sensitive. In order to find a solution to this problem new biomarkers of airway inflammation are being developed. YKL-40 is a chitinase-like protein that has a role in the inflammation and tissue remodeling in several human diseases. The aim of this study is to evaluate serum levels of YKL40 in children with intermittent or persistent asthma. We performed a cross-sectional analysis of serum samples from a cohort of patients with asthma and healthy controls. Patients with asthma were stratified according to four levels of asthma severity (mild intermittent, mild persistent, moderate persistent, and severe persistent). The analysis of serum samples was performed with the use of a commercially available enzyme-linked immune-adsorbent assay (ELISA) kit (Quidel). The minimum detection limit of the assay for YKL-40 is 15.6 ng per milliliter (ng/ml). Our data showed that circulating YKL-40 levels are significantly higher in patients with asthma than healthy subjects (36±18.6 vs 14:41±2.88, p= 0.001). Furthermore, we found significantly higher values of YKL-40 in both groups of children with intermittent asthma (p<0.001) and persistent asthma (p<0.001) than healthy controls. However, no correlation was found with duration and severity of asthmatic disease (r = 0:18, p= 0:33, r = 0.28 P = 0:13, respectively). Our data allow us to suggest that YKL-40 represents a useful biomarker of asthma in children with intermittent or persistent asthma.

Asthma is one of the most common chronic diseases in children. It is estimated that the number of people in the world suffering from asthma ix approximately 300 million, representing a major cause of hospitalization, access to the emergency room and school absence (1-4). To date, the diagnosis of asthma is mainly clinical, based on the clinical history, a careful physical examination and lung function tests. However, symptoms are often not specific and lung function tests are not very sensitive (5-8). Unfortunately, it has not yet been possible to translate this increased awareness of asthmatic disease in a better control of symptoms and a reduction of morbidity in children. Biomarkers of disease in asthma phenotypes are therefore needed to assess the inflammatory patterns, the different phenotypes, and the optimal response to therapy in order to improve the final outcome of these patients.

Key words: YKL-40; asthma; children; serum biomarker

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Recently, several biomarkers of airway inflammation have emerged as potential new tools for the diagnosis and management of asthma (9-11). Chitinases are enzymes that catalyze the hydrolysis of the β(1®4) glycosidic bonds in chitin. Mammals do not produce a chitinous structure, but they are able to express chitinases. The mammalian chitinases belong to the family 18 of glycosil hydrolase (GH18), and can be further subdivided into two broad categories: true enzymatically active chitinases and chitinase-like proteins that have no enzymatic activity, but are able to bind to chitin with high affinity (12, 13). YKL-40 (also called chitinase 3-like-1 or human cartilage glycoprotein 39) is a chitinase-like protein that is known to have a role in inflammation and tissue remodeling of several human diseases, including asthma (14). Interestingly, some gene variant chitinase have been recently identified to be associated with childhood asthma (15), further corroborating the evidence of a gene-environment interaction in childhood asthma (16-18).

There are few studies conducted on asthmatic children in order to clarify the role of this protein in bronchial inflammation during pediatric age and their results are still controversial. The aim of this study is to evaluate serum YKL-40 levels in children with asthma and to establish its clinical significance.

MATERIALS AND METHODS

We performed a cross-sectional analysis of serum samples from a cohort of children with asthma and healthy controls. Our study was approved by the local Ethics Committee of the Medical University of Catania and informed consents were obtained from parents of all patients. We consecutively enrolled patients attending the Bronchopneumology and Cystic Fibrosis Unit of the University of Catania and to Genetics and Pediatric Immunology of the University of Messina presumptively diagnosed by a physician to have allergic asthma between October 2013 and February 2014. According to international authors (1), the diagnosis of allergic asthma was based on clinical history, symptoms and signs, basal lung function tests, methacholine bronchial provocation test and specific allergen measurement by skin prick testing (LofarmaSpA, Milan, Italy) and/or the Immuno-CAP test. We included patients aged 6–16 years with a documented sensitization to 1 or 2 perennial allergens such as dermatophagoides dust and/or Parietaria pollen. Patients sensitized only to seasonal allergens, poly-sensitized patients and those who had previously undergone treatment with specific immunotherapy were excluded. The coexistence in the same patient of allergic rhinitis, diagnosed according to the latest international guidelines (19), was accepted. Patients with asthma were stratified according to four levels of asthma severity (mild intermittent, mild persistent, moderate persistent, and severe persistent) as defined by the frequency of asthma symptoms during the day, frequency of night-time asthma symptoms and measures of lung function (1). We excluded asthmatic children if they had been seen within the previous month at an emergency department visit or hospitalized for asthma or an exacerbation of asthma requiring systemic corticosteroids. During the period specified 30 patients meeting the inclusion criteria set out in the study protocol were enrolled. The control samples consisted of 21 healthy subjects with numerical and demographic characteristics similar to those of the study population. The analysis of serum samples was performed with the use of a commercially available enzyme-linked immune assay (ELISA) kit (Quidel). The minimum detection limit of the assay for YKL-40 is 15.6 ng per milliliter (ng/ml).

Statistical analysis of data was performed using SPSS software version 17.0 (SPSS Inc. Chicago, IL, USA). The results for the quantitative variables were expressed as mean ± standard deviation (SD), while qualitative variables as frequencies and percentages. Categorical variables were compared using the *chi*-square test, and the *t*-test for independent data was used to compare continuous variables. In the case of non-normal distribution the Mann-WhitneyU test was used. Simple associations were assessed with the use of Spearman's rank-correlation analysis. Multivariable linear regression analysis was performed to determine the

influence of variables on YKL-40 levels. Statistical significance was defined as p <0.05.

RESULTS

We studied a total of 30 patients with asthma (10 females, mean age 10.2±3.06 years, mean disease duration 2.5±1.87). Among patients in the study 20 (66.6%) had mild intermittent asthma, 7 (23.3%) mild persistent asthma and 3 (1%) moderate persistent asthma. A control population of 21 subjects (14 females, mean age 9.5±3.28 years) was also studied. The two groups did not differ regarding distribution in age and sex.

Twenty-four asthmatic patients out of 30 (80%) had a comorbid allergic rhinitis, while 6 patients (20%) had asthma alone; both subgroups, however, were homogeneous for demographic characteristics (age, sex), duration and severity of asthmatic disease (Table I). In Table II YKL-40 values are reported in children with different phenotypes of asthma and healthy controls. Our data show that circulating YKL-40 levels are significantly increased in patients with asthma than in control subjects (36±18.6 *vs* 14:41±2.88, P = 0.001). The multivariable linear regression analysis showed that factors considered confounders, including age and sex did not influence circulating YKL-40 levels (P>0.05). No statistically significant difference in serum levels of YKL-40 was found between patients with asthma and rhinitis compared to patients with asthma alone (38.04±14.6 *vs* 27.8±11.05, P = 0.186). Correlation analysis performed on the total samples of asthmatic patients between YKL-40 levels and duration and severity of disease, showed no correlation with either the duration (r = 0:18, P = 0:33), or with the degree of asthmatic disease (r = 0.28 P = 0:13). Moreover, we found significantly higher values of YKL-40 both in children with intermittent asthma (p<0.001) and in children with persistent asthma (p<0.001) than healthy controls, but not a significant difference of YKL-40 between intermittent asthmatic children and persistent asthmatic children (33.1±15.5 *vs* 40.7±8.3, p<0.07). No correlation was found between circulating levels of YKL-40 and the broncho-provocation test with methacholine (r =

Table I. *Demographic and clinical characteristics.*

	Asthmatics (n = 30)	**Controls (n =21)**	**P-value**
Sex_(m:f) (%) [A]	10:20 (67/33.0%)	9:12 (58/42.8%)	0.563
Age_(y) mean+/-SD [B]	10.2+/-3.06	9.5+/-3.28	0.460
Duration of disease (y) mean (SD)	2.5 (1.87)		
Intermittent Asthma (n.patients) **Mild Persistent Asthma (n.patients)** **Moderate Persistent Asthma (n.patients)** **Asthma and Rhinitis (n.patients)** **Asthma (n.patients)**	20 7 3 24 6		
PD_{20}–μg mean (SD)	457 (618.74)	-------	-------

Abbreviations: SD, standard deviation; PD_{20}, provocation dose of methacholine causing a fall in FEV1 of 20% A. Chi-Square Test; B. Student t-test for independent data.

0.15, P = 0:51). No difference was found between levels of YKL-40 and PD20 < 100 or PD20> 100.

DISCUSSION

The first clinically relevant data regarding the role of chitinases in asthma were reported by Zhu et al. (20). The authors found an excessive amount of AMCase in epithelial cells and macrophages in lung biopsies taken from patients with asthma. In line with these observations, high levels of AMCase were also found in the tears of subjects with ocular allergies (21) and in patients with chronic rhino-sinusitis and nasal polyps (22).

More recently, the levels of another chitinase-like protein, known as YKL-40, have been found increased in serum and lung tissue of patients with asthma (10). In addition, it has been recently demonstrated that the gene CHI3L1 coding for YKL-40 is highly heritable and it is associated with susceptibility to asthma (23). These results seem to indicate that YKL-40 is related to asthmatic disease, but the exact role still remains to be established, especially in children (24). In this regard, our study adds further evidence to the hypothesis that YKL-40 could represent a useful biomarker of bronchial disease even in children. Our data, in fact, show that circulating YKL-40 levels are significantly increased in pediatric patients with asthma compared to a control population. Confounder factors, including sex and age, do not affect the circulating levels of YKL-40. The presence of allergic rhinitis in comorbidity with asthmatic disease does not seem to affect the levels of the protein: no significant difference in serum levels of YKL-40 was found in patients with asthma and rhinitis compared to patients with asthma alone. This seems to suggest that the increase of the protein is a defining characteristic of patients with asthma. Higher values of YKL-40 were found even in children with intermittent asthma compared to healthy subjects and, therefore, it seems a very sensitive biomarker of disease in pediatric age such as serum IL 23 (25-28) or forced expiratory flow at 25-75% (29). No correlation was found

Table II. *YKL-40 in asthmatic children with different phenotypes and healthy controls.*

	YKL-40 values		**P-value**
Asthmatic children[a]	36 (18.6)	a--->a1	<0.001
Healthy controls[a1]	14.4 (2.8)	b---> a1	<0.001
Intermittent asthma[b]	33.1 (15.5)	b--->b1	ns
Persistent asthma[b1]	40.7 (8.3)	b1--->a1	<0.001
Asthmatic and rhinitis patients[c]	38 (14.6)	c--->c1	ns
Asthmatic patients[c1] **PD_{20}–μg mean (SD)<100**[d] **PD_{20}–μg mean (SD)>100**[d1]	27.8 (11.05) 32.4 (12.08) 40.8 (14.3)	d--->d1	ns

Abbreviations: SD, standard deviation; PD_{20}, provocation dose of methacholine causing a fall in FEV1 of 20%

between YKL-40 levels and duration and severity of asthmatic disease nor with lung function. A possible explanation for this could be that in our study we recruited mainly pediatric patients with a relatively short duration of the disease and with a degree of mild asthma whose lung function has not yet been compromised. We might speculate that in our study a correlation was not found with the severity of asthmatic disease because we did not include patients with severe persistent asthma who probably present a certain degree of tissue remodeling. However, there are in literature only two studies that investigated the role of YKL40 as biomarker of tissue remodeling in asthmatic children and results remain controversial (30-32). In conclusion, our data show that serum YKL-40 values are increased in serum of children with asthma compared to healthy controls, confirming its role as a biomarker of disease even in pediatric age. Additionally, in order to further confirm the pro-inflammatory role of YKL-40 in different inflammatory lung diseases, it could be useful to compare serum YKL-40 levels with other critical molecules (including High Mobility Group protein B1) involved in the same disorders (33-37).

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SUBLINGUAL IMMUNOTHERAPY IN CHILDREN: STATE OF ART

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Allergic immunotherapy (AIT) today represents a therapeutic practice for the treatment of allergic diseases such as rhinitis or asthma and is recognized as the only treatment able to modify the natural history of the disease. Administering gradually increasing doses of the causative allergen, AIT, has the objective of achieving immune tolerance against allergens. One of the administration routes most used in clinical practice is represented by the sublingual route. Current research on sublingual immunotherapy (SLIT) is focused on confirming the efficacy for all the different relevant allergens, on a better definition of allergen extracts and the improvement of their immunological properties and safety, on the identification of best treatment regimens, and on the possibility of extending the clinical indications. The aim of this review is to describe the most recent step in the field of SLIT development.

SLIT and specific mechanisms of action

The mechanism of action of AIT is characterized by a complex interaction between innate and adaptive immune response involving cells, cytokines and chemokines (Table I). SLIT is able to modify the presentation of the allergen by Dendritic Cells (DC) which coordinate the switching of the phenotype of allergen-specific T cell from a Th2 response, typical of allergic inflammation, to Th1 phenotype. A crucial role is played by interleukin (IL)-10, transforming growth factor beta (TGFß), and Th 17 cells (1). The immunologic cascade, in normal conditions, is activated by a low and repeated allergen exposure. This, in atopic subjects, induces a Th2 response that promotes the production of IgE by B cells and activates mast cells, basophils, and eosinophils, which in turn release inflammatory mediators (1). In SLIT, the high-dose allergen sublingually administered, leads to an immune deviation from a Th2 to Th1 response characterized by a release of Th1 cytokines such as IFNγ and the induction of Tregs suppresses the activity of Th2 cells and increases IgG and IgA production rather than IgE. The most important role is played by IL-10 Tregs and by TGFß Tregs (1, 2). IL-10 is a general inhibitor of proliferative response in T cells, which mediates the anti-inflammatory response by the activation of receptor-associated JAK family and STAT1, STAT3 and STAT5 (3-4). In response to an excessive or antigenic exposure, the overproduction of IL-10 acts by promoting peripheral T-cell tolerance (5), leading to and maintaining a state of anergy (6). The production of IL-10, during SCIT, is associated with a reduction of Th2 cells and Th2 cytokines (IL-4, IL-5 and IL-13) (7). The peripheral tolerance, during immunotherapy, seems induced by autocrine production, specifically the intracellular synthesis is increased in CD4 T lymphocytes (8, 9). The mechanism of action of TGFß, through a dependent and/or independent Smad pathway,

Key words: atopy; sublingual immunotherapy; safety; children

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during SLIT as well as in healthy patients, induces T-cell suppression (10). The action of TGFß is still mediated by a costimulation of CD28. CD28 molecule, indeed, allows to TGFß to inhibit T-cell receptor-stimulated proliferation of naive T cells by inducing apoptosis. The cooperation of CD28/ TGFB downregulates serum IL-2, IL-12 and IFNγ levels (11). Ciprandi et al. have shown that the production of TGFß significantly increases both after the first and after the second cycle of SLIT (12). IL-17 plays an important role in the activation of the immune system against an allergen. It has a proinflammatory role mediated by its activation from Th17. It has been shown that at two years of SLIT there is a great reduction in both symptoms and IL-17 production, explaining the fact that immunotherapy downregulates the release of IL-17 (13-16). At the start of SLIT, there is a shift of CD4 T cells from a Th2 (producing IL4 and IL5) to a Th1 profile (producing IFNγ Tregs and IL-10 Tregs) mediated by allergen stimulation (16-19). Tregs have not only the ability to induce the suppression of the Th2 response but also act on DC, Mast cells, Basophils, Eosinophils, and on the production of IgE, IgG and IgA (20, 21). In fact, in patients treated with SLIT, the B cells are stimulated to produce IgG4 and IgA2 antibodies that are able to block the activation of IgE and the subsequent activation of basophils and mast cells (22, 23). Another key role is played by memory FoxP3 Tregs, indeed the increase of FoxP3-expressing CD4 CD25 Tregs was found in the nasal mucosa of patients with grass pollen AIT, this was associated with a reduction of local allergic symptoms (24, 25).

SLIT IN YOUNG CHILDREN

Definition of SLIT patient selection

To be eligible for SLIT, patients should have a history of symptoms related to allergen exposure documented positive allergen-specific IgE test. Thus, the allergen used for AIT must be clinically relevant. Age does not appear to be a limitation. In fact, SLIT can be initiated in children under 5 years of age. Thus, the SLIT should start as soon as possible to the onset of the first allergic symptoms in order to obtain the best results (26). Therefore, although AIT may be considered as initial treatment, SLIT may be particularly indicated in: uncontrolled allergic patients with optimal pharmacotherapy, infants in whom pharmacotherapy causes undesirable side effects, children who are not constant and/or refuse long-term pharmacotherapy. In fact, it has been reported that, although the percentage of adherence should be regarded as satisfying, approximately 45% of children drop out of treatment and this trend is most common in children under the age of 4 years with a prevalence in the first 3 months of life (27). The main causes of treatment discontinuation in children under the age of 4 years can be attributed to discomfort in taking sublingual tablets/drops and the low palatability of the drug itself (28). In conclusion, the indications to treatment should be also based on the ability of the physician to correlate the clinical presentation with specific allergy testing, the severity of the allergic disease, and risk/benefit ratios (27).

Safety of SLIT

Generally, SLIT is a clinical practice that provides a safety profile both for the physician, who performs the operation, and for the patient. Moreover, SLIT does not require invasive procedures (such as the use of needles or infusions of intravenous drugs) and it can be comfortably managed at home, without a continuous medical supervision. Recently, in a comprehensive review of 104 articles on SLIT, 66 studies provided information on safety and tolerance of SLIT (28). However, although SLIT is well tolerated, side effects are also reported. The incidence of local side effects (itching and swelling of the lips and floor of the mouth, gastrointestinal disorders) has been estimated on average about 35%, but generally it is observed in up to 85% of patients in clinical trials (29). Usually, local side effects appear within minutes or hours after intake and are of short duration (less than 14 days), frequently self-resolving or requiring dose adjustment (30-32). Systemic reactions, including urticaria, angioaedema and asthma, may occur more frequently during dose escalation. No fatal events have ever been described but literature quotes twelve cases of anaphylaxis to SLIT. Risk factors for the occurrence of SLIT severe adverse events have not yet been established,

although it has been hypothesized that patients who have had previous systemic reactions to SCIT might have a higher risk (33). Additionally, height of season (34), history of previous systemic reactions (35), dose (36), and accelerated schedules (37) could also contribute to onset of side effects. Although side effects may be dose-dependent and allergen-dependent (38), to date, a dose-response relationship for safety is not clearly defined. It must be also considered that, in most cases, the accuracy of the adverse effects reporting depends on the patient's and/or family's recall and interpretation of the event. In the light of this, authors assessed the need for a generally accepted system of reporting SLIT adverse reactions that is applicable to both clinical practice and research (33).

CONCLUSIONS

Recent literature has shown that SLIT is able to change the history by induction of tolerance to the allergen. SLIT is able to inhibit the inflammatory cascade through the suppression of the Th2 response and increased IgG4 and IgA2. This mechanism is guaranteed by the release of immunologic mediators such as IL10 and TGFß and the inhibition of pro-inflammatory interleukins such as IL17. Despite the immunological framework being fully described, further studies will serve to relate those immunological mechanisms to abnormal function of airway epithelium in asthma (39-44). Future studies are needed to assess the interaction between the genetic predisposition to atopy and the induction of tolerance with immunotherapy (45-48).

Table I. *Mechanisms of sublingual immunotherapy.*

SLIT suppresses allergic TH2-mediated inflammation
SLIT early increases antigen-specific IgE
SLIT increases antigen-specific IgG, probably by induction of Tregs, immune deviation (Th2 to Th1), apoptosis of effector memory Th2 cells
SLIT is associated with persistent increases in antigen specific IgG4 and IgE blocking activity
SLIT inhibits eosinophils and reduce adhesion molecules in target organ
SLIT is associated with detection of CD25+ FOXP+ phenotypic Treg cells in the sublingual mucosa
SLIT induces long-term remission after discontinuation and may prevent new sensitizations

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VITAMIN D AND BRONCHIAL INFLAMMATION IN ASTHMATIC CHILDREN

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The role of the vitamin D in calcium homeostasis and bone metabolism is well known. In recent years it has been recognized that in addition to the traditional functions, vitamin D modulates a variety of processes such as host defense, inflammation and immunity. Epidemiological data indicate that low levels of vitamin D in serum are associated with impaired lung function and increased incidence of inflammatory diseases, infectious diseases and cancer. The authors studied the correlation among vitamin D levels, allergic inflammation, lung function and control of asthma and found a significant decrease of FeNO values (p= 0.0018) in children with vitamin D levels >30 ng/ml. These findings confirm that vitamin D plays a major role in bronchial inflammation.

Asthma is the most common chronic disease of childhood (1) however the role of genetic susceptibility and environmental factors is not yet well understood (2). Asthma control requires accurate diagnosis (1), evaluation of pulmonary function by spirometry (3-6), evaluation of bronchial inflammation by exaled nitric oxide (7-19) and assessment of coexisting conditions as atopy (20-22), allergic rhinitis (23-26) and atopic dermatitis (27). Cystic fibrosis (28) and other conditions that mimic asthma should be excluded. Asthma therapy includes quick-relief medications to control asthma symptoms (29) and Long-term asthma control medications (30-32). Recently new therapeutic approaches were tried in the treatment of asthma as probiotics (33), already used in intestinal infections (34) and vitamin D.

The role of the vitamin. D in calcium homeostasis and bone metabolism is well known. In recent years it has been recognized that in addition to the traditional functions, the vit. D modulates a variety of processes such as host defense, inflammation and immunity (35). Epidemiological data indicate that low levels of vit. D in serum are associated with impaired lung function and increased incidence of inflammatory diseases, infectious diseases and cancer (35-39). A number of recent studies have shown low levels of vitamin D in children with asthma (40-43).

The aim of our study is to verify the correlation among vitamin D levels, allergic inflammations, lung function and control of asthma.

Respiratory function was measured by spirometry (3), allergic inflammation by FeNO (exhaled nitric oxide measure) (7), and asthma control was assessed by the Children Asthma Control Test (C-ACT) (www.asthmacontroltest.com).

MATERIALS AND METHODS

We selected 66 children, affected by mild to moderate bronchial asthma (45 males and 21 females), aged between 5 and 19 years old, in the Pediatric Clinic of the Second University of Naples.

Keyword: asthma, FeNO, C-ACT, vitamin D.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

The study was performed between september 2013 to may 2014. Asthma diagnosis was made by typical history and a positive bronchodilator response and/ or bronchial hyperresponsiveness to methacholine (1). All the selected subjects were sensitized to house dust mite and did not use steroids for at least two weeks. Albuterol as needed was allowed. After the run-in period of two weeks all the children were randomly divided into two groups: children (N° 46) with serum levels of vitamin D < 30 ng/ml (not sufficient) (group 1) and children (N° 20) with serum levels of vitamin of vitamin D > 30 ng/ml (group 2). After the run-in period all the children were subjected to: i) determination of serum levels of vit.d. ii) assesment of exhaled nitric oxide (FeNO). iii) spirometry with bronchodilator response. iiii) C-ACT questionnaire. Spirometry was performed by a dry spirometer (Sensor Medics Vmax) according to ATS/ERS guidelines guidelines (3). FeNO was measured by single-breath on line method using an electrochemical device (MediSoft-Expair), according to ATS/ERS guidelines (7). The C-ACT questionnaire has been administered to children and their parents independently.

Statistical analysis

A descriptive analysis of examined population was carried out, expressing values as average ± standard deviation. Correlations were analyzed by spearman index (r), used for independent, quantitative variables of non-parametric data. Variables appear significant if $p < 0.05$. $r > 0.8$ very strong correlation; from 0.6 to 0.79 strong ; from 0.4 to 0.59 moderate; from 0.2 to 0.39 weak; <0.2 very weak.

RESULTS

Group 1 subjects (Vitamin D levels < 30 ng/ml) and group 2 subjects (Vitamin D levels > 30 ng/ml) have been compared at the beginning of the study to show any possible incoherence between the two groups and there was no statistically significant differences with respect to sex and age between the two groups. The FEVI values, the C-ACT values and the FeNO values have been compared between the two groups. With regard to respiratory function, no significant difference in the FEVI values has occurred between the groups. Children asthma control test (C-ACT) has shown no statistically significant differences in the group with vitamin D levels > 30 ng/ml compared to the group with Vitamin D levels < 30 ng/ml.

Our data show a significant decrease of FeNO values (p= 0.0018) in the children with vitamin D levels > 30 ng/ml to mean a major role of vitamin D on bronchial inflammation (Table 1).

DISCUSSION

In our study we have found no significant variations of FEV1 value between the groups, These data prove a normal lung function in subjects with

Table 1: *Comparing between the two groups for the three variables tested: vitamin D, FeNO and C-ACT.Group 1: children (N° 46) with serum levels of vitamin D < 30 ng/ml (not sufficient) and Group 2: children (N° 20) with serum levels of vitamin of vitamin D > 30 ng/ml. Values are expressed as mean ± standard deviation.*

Parameters	Group 1 (46 paz.)	Group 2 (20 paz.)	P* Value
Vit D (ng/ml)	21,1 ± 6	32 ± 1	<0,000001
FeNO ppb	32,3 ± 3,8	10,1 ± 3,6	0,0018
C-ACT	20,3 ± 4,3	20,9 ± 3,6	0,9

** test U di Mann Whitney*

insufficient vitamin D levels (vit. D < 30ng/ml) too.

Tolpannen et al (44) showed that lower vitamin D levels are not associated to lower lung function. On the other hand, Ayasin et al. (45) established a direct association between vitamin D levels and FEV1 in 50 asthmatic children and 50 healthy controls, aged between 6 and 18 (mean age 9.3) years. This study's results of the present study demonstrate that low FEV1 values are a significant predictors of low vitamin D levels. Probably the conflicting results on vitD and lung function association, depends on the differences in the design of the study and the severity of asthmatic symptoms. Chinellato at al (46) demonstrated that hypovitaminosis D is associated with low asthma control assessed by C-ACT. On the other hand, our study shown no significant association between vitamin D levels and C-ACT. Most likely, the differences in the age range of the selected children are crucial. In fact, in Chinellato's study 75 children, aged between 5 and 11 (mean age 9.6 ± 1.7 years) were examined, instead in our study, we have recruited 66 children aged between 4 and 19 (mean age 9.8 years).

Finally, we investigated the relationship between FeNO and vitamin D levels. It's widely demonstrated that inflammation plays a major role in the pathogenesis of bronchial asthma, and our data show that vitamin D levels >30ng/ml in asthmatic children are related to decrease in bronchial inflammation (Table I). Instead, Chinellato at al.(45) have not find any vitamin D effect on FeNO values and any significant correlation between FEV1 and vitamin D levels.

CONCLUSION

Our data show that vitD plays a role in bronchial eosinophil inflammation assessed by FeNO in asthmatic children. Further studies are needed to confirm our data and to establish the role of this vitamin in the balance gene-environment .

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SERUM AND BAL YKL-40 LEVELS IN DIFFERENT INFLAMMATORY LUNG DISEASES: AN UPDATE

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YKL-40 (also called chitinase 3-like-1 or human cartilage glycoprotein 39) is a chitinase-like protein belonging to the family 18 of glycosyl hydrolase (GH18). This protein is involved in the inflammatory process acting as pro-inflammatory cytokine secreted by neutrophils, activated human macrophages, vascular smooth muscle cells and cancer cells. It has been shown that YKL-40 has a role in pathological tissue remodeling and development of fibrosis of several diseases. To date, the biological and pathophysiological function of YKL-40 protein in pulmonary diseases is still unclear. This review focuses on the role of YKL-40 in diagnosis and monitoring of different lung diseases in order to assess whether this protein could be used as biomarker of specific conditions featured by inflammation and fibrosis. A comprehensive review of the literature using PubMed database, with appropriate terms, was undertaken for articles in English published since 1997. The literature search was undertaken in October 2014.

YKL-40 (also called chitinase 3-like-1 or human cartilage glycoprotein 39) is a chitinase-like protein consisting of 383 amino acids with a molecular mass of 40 kilodaltons (kDa) and belonging to the family 18 of glycosyl hydrolase (GH18) (1, 2), acting as a pro-inflammatory cytokine secreted by neutrophils (3), activated human macrophages (2), vascular smooth muscle cells (4) and cancer cells (5). The gene encoding for YKL-40 is located on 1q31-q32 of the human chromosome and includes 10 exons (2). Although it lacks enzymatic activity and therefore is not able to catalyze the first phase of degradation of the chitin, it nevertheless retains the ability to bind with high affinity to the chitin, heparin and collagen regulating proliferation, differentiation and survival of stromal cells (6, 7). Moreover, it has been shown that YKL-40 has a role in pathological tissue remodeling and development of fibrosis of several diseases, such as severe asthma (8), liver fibrosis (9-11), rheumatoid arthritis (12) and cardiovascular diseases (13-15). However, the biological and pathophysiological function of YKL-40 protein in pulmonary diseases is to date unclear (16-18). This review focuses on the role of YKL-40 in diagnosis and monitoring of different lung diseases in order to assess whether this protein could be used as biomarker of specific conditions featured by inflammation and fibrosis. A comprehensive review of the literature using PubMed database, with appropriate terms, was undertaken for articles in

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YKL-40 in asthmatic disease

Asthma is a chronic inflammatory disease characterized by airway hyper-responsiveness that results in intermittent and reversible obstruction of the airways (19-23). Currently, it is believed that allergic asthma is associated with an imbalance between Th1 and Th2 lymphocytes. Th2 lymphocytes produce a number of cytokines that are involved in the initiation and maintenance of inflammation in the airways (24, 25). Interleukin (IL)-4 and IL-13 stimulate the activation of B lymphocytes and their differentiation into plasma cells for the production of immunoglobulin E (IgE). Already in 2001, Webb et al. (26) reported the up-regulation of a chitinase-like protein, YM2, in Th2 $CD4^+$ lymphocytes of mice in response to the signaling of IL-4 and IL-13. High levels of YM2 and YM1 have also been found in the bronchoalveolar lavage (BAL) fluid of mice treated with IL-13 by intra-tracheal. A few years later, Lee et al. (27) demonstrated that also YKL-40 functions involve the Th2-inflammatory pathway. In 2007, Chupp et al. (8) found that YKL-40 levels were increased in serum and in lung tissue of patients with asthma. In support of these observations, Kuepper et al. (28) presented data from a follow-up study, demonstrating significantly higher levels of YKL-40 in both serum and BAL of asthmatic patients after bronchial provocation with various allergens. More recently, Tang et al. (29) found a significant correlation between serum YKL-40 level and total serum IgE levels in asthmatic patients. On the other hand, Specjalski et al. (30) did not report a correlation between the serum YKL-40 level and asthma severity and total serum IgE level. In another study, the authors demonstrated a positive correlation between YKL-40 and asthma severity, especially in the acute exacerbation group, whereas the serum levels of this protein did not seem to correlate with total serum IgE levels and asthma duration in years (31). In 2012, Otsuka et al. (32) demonstrated, in asthmatic patients, that sputum YKL-40 levels positively correlated with disease severity and sputum neutrophil counts: these results could be in line with those obtained from Chupp et al. demonstrating that YKL-40 serum levels were increased especially in severe asthma, a disease phenotype frequently associated with a more pronounced neutrophilic than eosinophilic airway infiltrate (33, 34). Moreover, several studies in literature have investigated the association between single nucleotide polymorphisms (SNP) in CHI3L1 gene and susceptibility to asthmatic disease. In this regard, in 2008 Ober et al. showed that the SNP rs4950928 in the promoter region of the gene was responsible for a perturbation of the binding site of transcription factors and consequently for a reduction in expression of YKL-40 (35, 36). According to this discovery, in 2009 Rathcke et al. (37) found a strong association between SNP rs4950928 G and asthma susceptibility. Although the link has not been demonstrated with asthmatic disease, two other SNP, rs10399805 and rs2275353, in CHI3L1 were found be significantly associated with atopy in a Korean population (38). In 2012 Verlann et al. (39) found that SNP rs103999331 and rs4950925 are able to modulate CHI3L1 expression and susceptibility to asthmatic disease in Caucasian and African populations. More recently in 2014 Tsai et al.d emonstrated that CHI3L1 polymorphisms rs1538372 and rs10399931 could be used as genetic markers predicting asthma risk in the Asian population (40).

YKL-40 in COPD

Chronic obstructive pulmonary disease (COPD) is featured by a progressive and irreversible airflow limitation (41, 42) and systemic inflammation (43). In this setting more attention has been given to YKL-40 protein. In 2008 Letuve et al.found increased concentrations of YKL-40 in plasma and bronchoalveolar lavage fluid from patients with COPD (44). The same authors demonstrated in COPD patients that alveolar macrophages exposed to YKL-40 are able to produce high levels of several pro-inflammatory biomarkers, such as IL-8, MCP-1, MIP-1α, and MMP-9 (44), and YKL-40 is also secreted from alveolar macrophages in response to TNF-α. In 2011, Sakazaki et al. (45), showed that serum YKL-40 levels are positively correlated to low-attenuation area percentage, a marker of the extent of lung emphysema, and negatively correlated

to lung function tests (FEV1) in patients with COPD. In a recent study conducted in 2012 by Gumus et al. (46), the authors found that serum YKL-40 correlated inversely with FEV1 (% predicted) and PaO_2 levels and directly with desaturation during a six minute walking test (6MWT). On the basis of these findings the authors suggested that high YKL-40 levels are related to hypoxemia and could be a marker of systemic inflammation in COPD. In a 10-year follow-up study, Holmgaard et al. (43) found that elevated serum YKL-40 levels were associated with increased mortality in this group of patients, supporting the notion that YKL-40 protein reflects an increased inflammatory response and a rapid decline of lung function.

YKL-40 in cystic fibrosis

Cystic fibrosis (CF) is a multisystem condition but progressive lung disease which results in respiratory failure is the main cause of morbidity and mortality. Persistent lower airway inflammation is further aggravated by intermittent episodes of acute worsening of symptoms, which are defined as "pulmonary exacerbations". Inflammation in CF is characterized by neutrophilic infiltrates with impaired antibacterial killing and proteolytic destruction of pulmonary tissue (47). Based on previous studies showing that neutrophilic cells secrete YKL-40 (48), some authors have hypothesized that YKL-40 could play a key role in CF lung disease. In this regard, Hector et al. (49) in 2011 quantified YKL-40 protein levels in serum and sputum supernatants from individuals with CF and healthy controls, assessed the breast regression protein of 39 kDa (BRP-39) levels, a murine homologue of YKL-40, in a mouse model of CF-like lung disease and evaluated the impact of SNP in CHI3L1 gene on CF lung disease severity. The authors found increased YKL-40/BRP-39 levels in both sputum supernatants and serum from CF patients and a mouse model of CF-like lung disease. Moreover, it was demonstrated that among CF patients, YKL-40 levels were significantly higher in sputum, where they correlated positively with numbers of neutrophils, than in serum. In addition a positive correlation was found between YKL-40/BRP-39 airway levels and airflow obstruction in pulmonary functions tests. This study also demonstrated that two YKL-40 SNPs, rs871799 and rs880633, modulated age-adjusted lung function in CF patients. Based on these findings, the authors suggest YKL-40 levels in sputum as potential biomarker of neutrophilic airway inflammation in CF. Recently, Fantino et al. (50) found that YKL-40 in BAL correlated with pulmonary infection with *P. Aeruginosa* and BAL markers of inflammation (macrophage and neutrophil number, neutrophil elastase, CXCL8, IL-1β). On the other hand, serum YKL-40 levels did not correlate with BAL levels in the same individuals or with inflammatory markers. Furthermore, YKL-40 was below the limit of detection in urine (30 pg/ml). The authors concluded that levels of YKL-40 in BAL reflect airway inflammation and infection in early CF lung disease, but the lack of increased YKL-40 in serum in the absence of systemic inflammation limits the benefit of this potential biomarker in early disease.

YKL-40 in other lung diseases

Idiopathic pulmonary fibrosis (IPF) is a progressive disease characterized by remodelling of the lung tissue. Although its etiology is to date unclear, lung remodelling seems to be attributed to chronic inflammation, fibroblast proliferation and pneumocyte dysfunction (51-53). In 2010 Furuhashi et al. (54) found elevated YKL-40 levels in the serum and lungs of patients with IPF and demonstrated by immunohistochemistry that, in these patients, YKL-40 was expressed by alveolar macrophages and alveolar epithelial cells near fibrotic lesions. In addition, the same authors found an inverse correlation between serum YKL-40 and lung function in IPF patients (54). In 2011, Korthagen et al. (55) found serum and BALF YKL-40 levels significantly higher in IPF patients compared to healthy subjects; and the highest levels of YKL-40 in serum and BALF were associated with a shorter survival time. The authors also demonstrated that BALF YKL-40 levels were genotype-dependent, although the presence of the rs10399931 polymorphism in CHI31L did not influence the susceptibility to IPF disease. YKL-40 is also produced by cancer cells and could have a role in angiogenesis and cancer cell proliferation

(56-59). In relation to this, the authors demonstrated that elevated pretreatment serum YKL-40 levels in patients with non-small-cell lung cancer (NSCLC) were an independent prognostic variable of poor survival (57, 58). Thom et al. demonstrated that the pretreatment serum YKL-40 level had no influence on response to chemotherapy in NSCLC (59). However, Xu et al. (60) reported that higher serum YKL-40 in SCLC patients were associated with a lower response to chemotherapy. These different results could find an explanation in different mechanisms of carcinogenesis between NSCLC and SCLC.

New data have emerged in literature on the role of YKL-40 in infectious disease, including community-acquired pneumonia (CAP). In 1999 Nordenbaek et al. (61) showed that people hospitalized with acute CAP had elevated serum YKL-40 concentrations. These authors found that the levels of the protein returned to the normal range within one week after antibiotic therapy. In line with these results, Wang et al. in 2013 (62) reported that patients with CAP had higher serum YKL-40 levels than healthy controls. The authors also demonstrated a significant correlation between the protein and several severity indices (PSI, CURB-65, APACHE II scores and length of hospital stay). Finally, controversial data exist also regarding the role of YKL-40 in predicting bronchiolitis obliterans syndrome (BOS) development after lung transplantation. In 2013 Kastelijn et al. (63) did not find any difference in YKL-40 concentrations between a BOS group and control group. However, further studies did not confirm these findings (64).

CONCLUSION

YKL-40 is a chitinase-like protein involved in several diseases featured by inflammation and tissue remodeling. Despite important advances in knowledge of chitinase-like proteins in recent years, the biological and pathophysiological function of YKL-40 in pulmonary diseases is to date unclear. Recent studies have shown increased YKL-40 levels in serum and BAL of patients with a wide variety of lung diseases. This heterogeneity could find a possible explanation in the fact that the protein is secreted by several cell types including neutrophils, activated human macrophages and cancer cells. Although the YKL-40 increase does not seem to be a specific biomarker of a single condition, however, data in literature support the hypothesis that high levels in serum and BAL could be useful in monitoring the evolution of several pulmonary diseases and represent a biomarker of poor prognosis. Additionally, to confirm the pro-inflammatory role of YKL-40 in different inflammatory lung diseases, it could be useful to compare serum YKL-40 levels with other critical molecules (including High Mobility Group protein B1) involved in the same disorders (65-68). Further studies are needed in order to better clarify the clinical setting of YKL-40 in severe pulmonary diseases.

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β2-AGONISTS IN CHILDHOOD ASTHMA

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β2-agonists reduce airflow limitation by improving airway diameter as a consequence of a direct action on airway smooth muscle. β2-agonists can be broadly classified according to their duration of action: short-acting β2-agonists (SABAs), including albuterol, terbutaline and fenoterol, have pharmacodynamic half–lives between 2 and 6 h and long-acting β2-agonists (LABAs), including salmeterol and formoterol, require twice daily treatment. SABAs are often used "as needed" for asthma exacerbations and before exercise in the presence of exercise-induced bronchospasm. LABAs provide longer symptom control, which is a particularly useful feature for preventing night-time symptoms. There are two main LABAs, salmeterol and formoterol. This review focused on the recent data published on this topic.

The beta2-adrenoreceptor is a large molecule of some 413 aminoacids. Most of the molecule is in the muscle cell wall, but it also has a tail outside the cell, and several loops within the cytoplasm. Adrenoreceptors are classified as β1, β2 or β3, identified in cardiac, airway smooth muscle, and adipose tissue, respectively. β2 receptors are found in increasing density with increasing airway generations to the alveoli (1). They are coupled to Gs protein, where stimulation by a β2-adrenoreceptor (AR) agonist activates adenylcyclase and increases cAMP levels. cAMP increases protein kinase A (PKA) activity, which phosphorylates downstream protein modulators (2). The overall activation of this signal transduction pathway can lead to the inhibition of phosphoinositol hydrolysis, a fall in intracellular $Ca2^{+}$ levels, and the activation of large conductance K^{+} channels. The hyperpolarization of airway smooth muscle as a result of opening K^{+} channels can lead to relaxation of airway smooth muscle (3).

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Short-acting β2-agonists (SABAs) SABAs are often used "as needed" for asthma exacerbations and before exercise in the presence of exercise-induced bronchospasm (5, 6).The widely used short-acting β2-agonist is albuterol, a hydrophilic molecule that accesses the receptor from the extracellular compartment and has a rapid onset of action. The lipophilic agonists have a longer duration of action because they burrow into the cell membrane (1). Albuterol has negligible α-AR activity at recommended clinical doses and demonstrates a substantially greater selectivity between β2- and

Key words: β2-agonists, Short-acting β2-agonists, Long-acting β2-agonists, asthma, children

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

β1-ARs than any other product previously available (7). Albuterol has approximately equivalent potency at relaxing human isolated bronchi *in vitro* and bronchodilator potency in asthmatic subjects compared with epinephrine (8) and, compared with isoproterenol, albuterol is at least as potent a bronchodilator, has a much longer duration of action, and is much less likely to influence blood pressure or heart rate (9). After inhalation of albuterol, maximum bronchodilation can be seen within 15 min of inhalation (10). However, albuterol binds only weakly to the receptor and quickly diffuses back into the microcirculation. This accounts for its short duration of action (4–6 h) (7). Nevertheless, because of its rapid onset of action, which is a clear clinical advantage for the reversal of bronchoconstriction, albuterol is usually considered the drug of choice as relief medication for symptoms of bronchospasm. Although pressurized aerosol and aerosol solution are effective means to deliver albuterol into the lungs, even in patients with poor ventilation (11), when the response to inhaled albuterol is reduced or absent, intravenous administration can be used as an alternative (12).The British Thoracic Society stated that continuous intravenous infusion should be considered when there is uncertainty about reliable inhalation or for severe refractory asthma. They recommend a 15-μg/kg bolus of intravenous albuterol early in treatment as an adjunct in severe cases (13).

The recent Global Initiative for Asthma guidelines include the use of intravenous beta2 agonists in patients with poor response (peak expiratory flow < 30% of predicted, severe symptoms, hypercapnia and hypoxemia), and to admit the patient to the intensive care unit (14).

LABAs provide longer symptom control, which is a particularly useful feature for preventing night-time symptoms (15). There are two main LABAs, salmeterol and formoterol. As a result of their structure, salmeterol and other LABAs are lipophilic and diffuse into the cell membrane. The "tail" binds into the beta receptor at an exosite. When the tail is in association with the exosite, the molecule isprevented from dissociating from the receptor. The "head" binds to AR and activates the beta2 receptor at the same place where albuterol binds. The head continually attaches and detaches from the receptor site, by the charniere (hinge) principle; the flexion is around the oxygen atom on the tail. This repeating attachment process prolongs the drug's action (16). Salmeterol is highly lipophilic and rather slowly diffuses through the lipid bilayer in muscle cell membranes to reach the beta2-receptors, explaining the slower onset and long duration of action (17). Formoterol, being less lipophilic, has a fast onset of action, similar to short-acting beta2-agonists, and it is believed to be incorporated into the lipid bilayer to serve as a reservoir, accounting for its prolonged action (18). LABAs (formoterol or salmeterol) plus low-dose inhaled steroids are indicated as the preferred controller treatment in step 3 of the asthma management plan (14). Concomitant products of LABA and ICS are available for use in children over 4 (salmeterol) and 6 (formoterol) years, however, the effect of LABAs or concomitant products has not yet been adequately studied in young children under 4 years of age (19). For over a decade, the FDA and the medical community have discussed how to safely use LABAs (20). Even before U.S. approval of the first LABA, there was concern about a paradoxical increase in serious asthma exacerbations in some patients treated with these drugs (21-23). The Serevent nationwide surveillance (SNS) study suggested an increased risk of asthma-related death in patients treated with salmeterol as compared with albuterol (24). Shortly after approving salmeterol in 1994, the FDA began to receive reports of serious asthma exacerbations and deaths in patients treated with the drug (25-27). To further evaluate these reports, the manufacturer (Astra-Zeneca) conducted the Salmeterol Multicenter Asthma Research Trial, in which there were eight more asthma-related deaths per 10,000 patients among those treated with salmeterol than among those given placebo (28). The authors compared the safety of salmeterol or placebo added to usual asthma care. There was a small, significant increase in respiratory-related deaths and asthma-related deaths or life-threatening experiences in subjects receiving salmeterol *vs* placebo. Smaller studies on the safety of formoterol showed an excess of serious asthma exacerbations, some necessitating

intubation (29). Although the absolute risk observed was small, it was not inconsequential, given the number of patients with asthma currently receiving these drugs. The FDA conducted a comprehensive review of the benefits and risks of using LABAs to treat asthma (20). The agency concluded that the benefits of LABAs continue to outweigh the risks when the drugs are used appropriately and that the agents should remain available for the treatment of asthma. However, because of their serious risks, the FDA recommended that LABAs should be reserved for patients whose asthma could not be adequately managed with asthma-controller medication such as an inhaled corticosteroid. Furthermore, until additional data were available from large, randomized, controlled trials evaluating the safety of LABAs when administered with an inhaled corticosteroid, the FDA believed that long-term use of LABAs should be limited to patients who required prolonged use of these drugs (20). There were several reasons for these label changes. Firstly, although it was already common practice that a LABA should not be used without concurrent use of an asthma-controller medication, the new contraindication emphasized the seriousness of the risk associated with LABA monotherapy. Secondly, the FDA believed that the risks of LABAs could be minimized if the agents were used judiciously and that patients who did not require long-term LABA therapy should not be exposed to the risk. Data indicated that many patients were administered the combination products containing an inhaled corticosteroid and a LABA without first undergoing stepwise increase in treatment with an inhaled corticosteroid alone. Thirdly, long-term use of LABAs in patients with asthma should be limited to those whose asthma could not be controlled with asthma-controller medications. Finally, the FDA recommended the use of combination products containing an inhaled corticosteroid and a LABA in children and adolescents because of the difficulty of ensuring compliance with both medications when they were administered separately in these groups (20).

Recent evidence

In patients with asthma, the persistence of inflammatory condition is associated with airway remodelling, characterized by structural changes in airway tissues such as epithelial goblet-cell hyperplasia, subepithelial collagen deposition, and smooth-muscle hypertrophy (30). These changes are associated with bad long-term clinical outcomes and have been attributed to eosinophilic inflammation (31). However, *in vitro* studies indicate that the compressive mechanical forces that arise during bronchoconstriction may induce remodelling independently of inflammation (32-34). In a rhinitis model too, proinflammatory cytokines arise (34).

More recently, to test this hypothesis in humans, Grainge et al. performed repeated inhalation challeng es with exposure to either allergen (to induce bronchoconstriction with airway eosinophil recruitment) or methacholine (to induce bronchoconstriction alone) in volunteers who had mild atopic asthma, and then they analyzed bronchial biopsy specimens obtained before and 4 days after completion of the challenges (36-40). The results showed that both challenges induced significant airway remodelling. The changes appeared to be independent of eosinophil recruitment into the airways and they were independent of the stimulus causing the bronchoconstriction. The link about atopy, autoimmunity and infections is debated (37-39).

These data show the potential contribution of airway narrowing to airway remodelling and these findings may have implications for the management of asthma. At present, the primary aim of asthma management is to control the disease by reducing airway inflammation.

The results of this study suggest that an additional target should be to stabilize airway caliber to prevent bronchoconstriction. These findings may have potential implications for management, in particular on LABAs treatment (36;40).

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ATOPIC DERMATITIS: MELATONIN AS POTENTIAL TREATMENT

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Atopic dermatitis (AD) is a chronic relapsing-remitting inflammatory skin disorder, characterized by a skin barrier dysfunction resulting in epidermal damage and altered permeability to allergens and microbes. Traditionally, the immunological mechanism involving the Th1-Th2 paradigm is considered central in the pathogenesis of AD. However, oxidative stress is, currently, recognized as a fundamental predisposing stimulus for AD. Several therapeutic approaches have been proposed as treatment, including the use of melatonin. This indolamine, through widespread expression and pleiotropic activity of the cutaneous melatoninergic system, may counteract environmental and endogenous stressors, regulate the immune response, decrease oxidative stress, and, finally, promote skin integrity. In the light of its pleiotropic effects, melatonin could represent a potential and alternative therapeutic approach in patients with AD.

The currently used term for eczematous hypersensitivity reactions in the skin, is "atopic eczema/dermatitis". This includes atopic/extrinsic, immunoglobulin (Ig) E-associated mechanisms, and non-atopic/intrinsic, not IgE-associated forms of these conditions (1).

Atopic dermatitis (AD) is a chronic relapsing-remitting inflammatory skin disorder, characterized by a skin barrier dysfunction which results in epidermal damage and altered permeability to allergens and microbes (2). Prevalence of this condition is on the increase and affects 15–30% of children and 2–10% of the adult population (3). AD is characterized by heterogeneous clinical manifestations including eczema, erythema, edema, excoriation, scaling, and itching. Based on the severity of the disease, and because of stress related to this chronic disorder and the impaired immune response, sleep disturbances have also been reported. Although the pathogenesis of AD is complex and still not fully understood, it has been hypothesized that genetic predisposition, environmental factors, and skin barrier dysfunction are involved.

Melatonin, (-acetyl-5methoxytryptamine), is an endogenously produced indolamine synthesized fromthe pineal gland and other organs (4). In mammals, melatonin is likely produced in every cells and its production in the skin is well documented (4).

Among a broad range of actions attributed to melatonin, its antioxidant and immunoregulatory properties may be useful in the treatment of AD (5-11).

Atopic dermatitis and immunity

The epidermis plays a protective role with its antimicrobial barrier, and as permeability barrier to

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retard transcutaneous evaporative water loss. When barrier is disrupted, for example, by environmental factors and/or the filaggrin gene mutation, it is predisposed to be penetrated by external stimuli (5-11). Foreign antigens, haptens and/or protein antigens, promote an abnormal immune response which, in turn, plays a critical role in the development, maintenance and flare-up of AD. Although first exposure to antigens initially induces T-helper (Th)1 cells, repeated exposure causes, firstly, a shift toward Th2-dominated responses and, secondly, in a shift toward Th1-dominated responses. Thus, based on the impairment of different T helper cells, as well as cytokine secretion profiles (8), it is possible to identify two critical intervals in immune-pathogenesis of AD: an acute and a chronic phase (8).

In the acute phase, naïve CD4+ T cells differentiate into prevailing Th2 cells which produce specific cytokines (12, 13). In particular, Th2-dependent cytokines include (i) IL-4, involved in IgE switching, (ii) interleukin (IL)-5, attracting eosinophils, (iii) IL-13 (14), and (iv) IL-31, a pruritogenic Th2 cytokine (15).

In chronic AD lesions, there is a switch to cytokines derived mainly from Th1 cells, including IL-2 and tumor necrosis factor-a (TNF-a). This leads to interferon (IFN)-α release which, in turn, inhibits IgE synthesis and Th2 expansion, but supports Th1-cell growth (16). Moreover, in the chronic phase, in addition to Th1/Th2 biphasic and distinct inflammatory responses, a currently accepted model proposes a mixed Th1/Th2 pattern, influenced by other T sub-populations such as T regulatory (T-reg) cells and Th17 cells (17). T-regs are heterogenic T cell populations with different surface markers; these suppress the activated immune system, and prevent autoimmunization (18).Although the published data are not completely clear on the role of T-regs in atopic diseases, it has been hypothesized that T-regs, in addition to T-helper and B-cells, are involved in the release of anti-inflammatory cytokines, such as IL-10 (19). T-regs diminish thr Th2 immune response, and target other cell types, including dendritic cells, basophils and eosinophils. Additionally, T-regs influence allergen-specific IgE regulation, inhibition of mast cell degranulation (20), and, finally, they are involved in the interaction with resident tissue cells and remodelling. Thus, lower T-reg cell levels, as reported in AD patients, may also explain the immune dysregulation detected in atopic subjects (20).

Th17, CD4 T effector subsets characterized by the production of pro-inflammatory cytokines (IL-21, IL-22, TNF-α, and IL-17) are also involved in the phenotypic expression of AD (21-24). In particular, major serum IL-17 levels have frequently been detected in the acute phase of AD and rarely in the chronic phase of the disease (25). Different levels of circulating Th17 probably reflect tissue infiltration of these cells in acute and chronic phases (25), which also explains the "multiple face" of AD (25, 26).

Finally, the cause of AD appears to involve a feedback cycle: mechanical damage leads to the production of pro-inflammatory cytokines, which recruit T-cells (initially a predominant Th2 response and later a predominant Th1 response) into the skin. The release of chemokines, in turn, promotes IgE production and systemic inflammatory responses, leading to pruritic inflammation of the skin, and, further to mechanical damage (27).

Atopic dermatitis and oxidative stress

Although the Th1-Th2 paradigm is considered central in the pathogenesis of AD, oxidative stress is also a fundamental predisposing stimulus in AD. The skin is exposed to endogenous and environmental pro-oxidant agents, leading to the generation of destructive reactive oxygen species (ROS), icluding free radicals. Generally, there is anequilibrium between free radical formation and endogenous antioxidant defence mechanisms (enzymatic antioxidants: glutathione peroxidase, catalase and superoxide dismutase (SOD); non-enzymatic antioxidants: α-tocopherol, β-carotene, ubiquinon, melatonin, ascorbate and glutathione). However, if the oxidant/anti-oxidant balance is disturbed, it can produce oxidative damage (28). AD is histologically characterized by infiltration of lymphocytes, monocytes and eosinophils. These cellsare hyper-reactive and show enhanced release of substances including pro-inflammatory cytokines and ROS, including oxygen (O2-), hydrogen peroxide (H2O2), and peroxinitrite, which, in turn, promote further oxidative damage and activation of an immune

status. Oxidative stress, which involves to proteins, DNA and membrane lipids, can also lead to cell death and stimulate an inflammatory response, thereby further advancing AD (29). Therefore, ROS can act as second messengers in the induction of a vicious cycle leading to further immune activation, oxidative damage, and, thus, propagation of a pro-oxidant status (30). ROS can also promote activation of cytokines and modulation of signalling pathways including nuclear factor-kB (NF-kB) or activator protein (AP)-1. Activation of NF-kB induces expression of pro-inflammatory proteins involved in immune response, including IL-1, IL-6, TNF-α, INF-α, as well as H2O2 (30). It has been suggested that up-regulation of AP-1 may be associated with a defect in ceramide generation which could result in enhanced protein kinase-C activation, leading to excessive release of pro-inflammatory cytokines by AD keratinocytes (30). Generally, peroxisome proliferator-activated receptors (PPARs), member of nuclear factor family, also influence the biological activity of keratinocytes. To be precise, PPAR isoform α (PPAR-α) counteracts the inflammatory response by inhibition of the expression of pro-inflammatory genes as well as cytokines and metalloproteases. PPAR-α activation also induces antioxidant enzymes (catalase, SOD) which would reduce the oxidative damage and the inflammatory response (31).

The anti-oxidant system, however, is also altered in AD keratinocytes. Additionally, it has been suggested that an impaired homeostasis of oxygen/nitrogen radicals associated with elevated oxidative stress, measurable as urinary 8-hydroxy-2-deoxyguanosine (8-OH-dG) excretion, a parameter of oxidative DNA damage, may be further related to the pathophysiology of AD (32). In children affected by AD, urine concentrations of 8-OH-dG, acrolein-lysine adducts (markers of lipid peroxidation), and bilirubin oxidative metabolites (markers of bilirubin oxidative metabolism) are higher than those of control subjects, and returned to normal values on disease remission (33). These findings suggest that increased oxidative stress, derived from both the increased production of ROS and altered antioxidant defences, contributes to the pathophysiologic mechanisms of AD. Evidence of enhanced protein and lipid-oxidative damage was found in AD patients, as demonstrated by the increase of carbonyl moieties both in lesional and non-lesional skin, along with an higher activity of SOD, an effective scavenger of ROS (34). In addition, experimental and human studies determined that the oxidative/anti-oxidative imbalance promotes a shift toward a Th2-skewed immune response, favouring the atopic status (35). In accordance with these data, the administration of anti-oxidants in AD patients might promote the resolution of the inflammatory condition and limit oxidative damage. It has been reported that N-acetyl-L-cysteine (NAC), a precursor of glutathione (GSH), promotes down regulation of Th2-cytokines (IL-4, IL-5 and INF-γ), leading to a subsequent over-expression of Th1, thereby inhibiting development of Th2 immune response (36).

Finally, oxidative stress promotes AD by acting both directly, through cellular damage, and indirectly, by the shift of the balance from Th1 towards Th2 cytokine profiles, bothin vitro (37) and in vivo model systems (38, 39). Therefore, by counteracting these pleiotropic effects,oxidative tissue injury could be reduced, and the humoral immune response in AD could be modified.

Melatonin in the skin

The skin represents a barrier between the internal milieu and the environment, preserving the underlying tissues from pathological agents. In addition to its mechanical function, the skin interacts with the neuro-endocrine system through specific messengers including melatonin, acting as a hormone, neurotransmitter, cytokine, growth factor, or biological modifier (40, 41). The melatonin/skin link has been well recognized since the initial identification of action of melatonin in lightening the skin pigmentation in frogs (42). In the skin, L-tryptophan is transformed into melatonin (43), via serotonin and N-acetylserotonin (NAS) intermediates. In particular, mammalian skin is capable of converting serotonin to melatonin through both classical arylalkylamine N-acetyltransferase (AANAT-dependent) and alternative (AANAT-independent) pathways (44, 45). The released melatonin acts on the skin cells in a receptor dependent and receptor independent manner. Human skin expresses melatoninspecific

receptors (MT1, MT2, MT3), as well as nuclear receptors of RZR/ROR subfamily of orphan receptors (45, 46) which, by binding with melatonin, have the potential to mediate phenotypic actions on cellular proliferation and differentiation (45, 46). Moreover, the widespread expression and pleiotropic activity of the cutaneous melatoninergic system produces a high level of cell-specific selectivity, ensuring adequate skin integrity and counteracting both environmental and endogenous stressors (47). In accordance with these findings, in animal models, it has been determined that long-term pinealectomy is associated with reducedskin thickness. Conversely, melatonin administration reverses the atrophic response of the epidermis, limits mitochondrial swelling and collagen fiber injury (48). Additionally, exogenous melatonin also inhibits cutaneous vascular permeability (49) and promotes angiogenesis and wound healing (50).

Melatonin also exhibits the following receptor-independent properties: (i) cytoprotection and/or metabolic modulation (51), (ii) antiapoptotic effects, (52) (iii) radical scavenger, (iv) inhibition of lipid peroxidation and nitricoxide formation (53), (v) stimulation of anti-oxidative enzymes, (53) (vi) and, finally, stabilization of mitochondrial membranes (53). Thus, also non receptor-mediated actions of melatonin play a critical role in preserving cell function/integrity/survival in maintaining functional epidermal barrier after its disruption by external or internal stressors and counteracting pathology at both local or systemic levels.

Melatonin and atopic dermatitis

Melatoninplays a crucial role in several important physiological functions including regulation of circadian rhythms, vascular, neuroendocrine, neurological, and immunological actions (54), andin cutaneous biology (55). Already, almost three decades ago it was proposed that melatonin, acting as a neuroendocrine factor, improves biological hypersensitivity to environmental triggers in AD (56). Later, melatonin is was found toinhibit inflammatory response associated with contact hypersensitivity (11), and, finally, to attenuate skin sympathetic nerve activity (SSNA) response to mental stress (57). AD patients are, in fact, subject to psychological stressors, which can aggravate AD (58). In particular, it is reported that levels of stress, anxiety and depression are higher in AD than in non-AD patients (59). Additionally, these psychological parameters, especially anxiety, are strongly associated with symptoms of AD(60). Disturbed sleep and, consequently, an impaired quality of life are also frequently reported by AD patients. Factors contributing to sleep disturbance include pruritus, scratching movements (61), atopic predisposition (serum total IgE levels) (62) and sensitization (serum specific IgE levels) (63). Melatonin levels in patients with AD have been found to be lower those in healthy subjects (56-65). Thus, indipendent of the physiological nocturnal peak of melatonin in the pineal gland, it has been hypothesized that a reduction in extra-pineal serum melatonin levels and partially reduced activity of the sympathetic nervous system, which is also involved in the control of melatonin secretion (56), contribute to sleep disorders in AD patients (66). In contrast, recently Chang et al. claimed that nocturnal melatonin levels were significantly higher in children with AD compared with controls. Moreover, a higher nocturnal melatonin level was associated with better sleep efficiency, less sleep fragmentation, and milder disease severity (66). They hypothesized that the elevated melatonin secretion was a compensatory response to sleep deprivation associated with AD (66). In children with AD, who presented a compensatory rise in blood melatonin levels, exhibited better sleep and an improved skin condition (66).

Sleep disorders can be a psycho-emotional stressor that elicits neuroendocrine stress reactions with consequences for the immune response and chronic inflammatory skin diseases (59, 67). Sleep deprivation promotes both an increased release of pro-inflammatory cytokines (IL-1, IL-6) and the reduced synthesis of regulatory molecules (IL-12) with an impaired antigen presentation pathway (67, 68). In AD patients, this abnormal immune response might be a further indication of the need for melatonin supplementation, since this indolamine may acts between the innate immune system and sleep circuit (69). In an animal model, it was reported that melatonin reduces serum IgE levels and inhibits IL-4 production by activated CD4+ T cells (70). Melatonin does not

have receptors on B cells and, therefore, does not boost "humoral" (Th2-type) immunity (71). However, both Th1 and Th2 express membrane receptor, MT1, and the nuclear binding site for the melatonin; the latter enhances the Th1 response thereby increasing the production of INF-ɣ and IL-2 and suppressing the synthesis of IL-10, via nuclear receptor-mediated transcriptional control (72, 73). However, this capacity is strongly reduced in AD patients who exhibit lower IFN-ɣ production (73), potentially leading to lower melatonin release which, in turn, may contribute to sleep disturbances and additional stress (74).

Melatonin, via specific receptors, also seems to prevent degranulation, infiltration and activation of mast cells in the dermis which normally contribute to skin injury (75). Furthermore, melatonin promotes antigen presenting cells (APCs) (monocytes and macrophages) activities and enhances their phagocytic capacity (76). Melatonin also enhances the production of IL-1, IL-6 and IL-12 in human monocytes. These mediators may counteract stress-induced immunodepression, showing an interesting immunotherapeutic potential against infections, especially in AD patients, which are susceptible to viral and bacterial colonization (77-79). Although melatonin does not have receptors on B cells, it can influence hemopoiesis by stimulating hemopoietic cytokines, of specific progenitor cells, such as pre-B cells, monocytes and natural killer (NK) cells (71), to further modify the immune response. It has also been reported that melatonin can act as a potential inducer of Th17 cell differentiation, influencing the onset and progression of immune-related morbidities, including atopic diseases (71). Finally, especially during stress or an immune imbalance, the synthesis of melatonin strongly influences the rhythmicity of the Th2/Th1 cell ratio, involved in the etiopathogenesis of AD, in an intracrine, autocrine, and/or paracrine manner (71).

Despite the fact that exogenous melatonin has been safely administered to adults and children (80, 81) to date, a randomized clinical trial, focusing on evaluation of both sleep quality and dermatitis severity after melatonin administration in AD patients has yet to be carried out. We believe that, in light of its antioxidant and immunomodulatory effects, as well as the absence of long-term toxicity, AD patients could benefit from melatonin supplementation (82).

CONCLUSIONS

AD prevalence has continued to increase. However, the exact pathogenetic mechanisms of this conditionare still not understood. Several therapeutic approaches have been proposed for the treatment of this disease. Melatonin, due to its pleiotropic activities which are mediated by both receptor-dependent and receptor-independent processes, affects skin morphology and physiology, counteracts environmental and endogenous stressors, and maintains cutaneous integrity. Moreover, melatonin, acting via the neuro-immune-endocrine system involved in the pathogenetic pathways of AD, might also modify the onset and progression of this disease. Additional studies are needed to understand the molecule-specific mechanisms of action of melatonin and to establish efficacy of this compound in human AD subjects.

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WALNUT SENSITIZATION IN PEDIATRIC AGE: A PRELIMINARY STUDY

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Walnut consumption has recently become a healthy dietary habit worldwide, due to its positive benefits in reducing cholesterol levels and oxidative stress; this has resulted in an increase in individual consumption, global production and risk of developing sensitization and allergy. In general, clinical manifestations of walnut allergy are frequently severe and systemic potentially life-threatening, leading to anaphylaxis both in the pediatric and adult populations. In light of these findings, we performed a preliminary study considering the walnut native allergen and the recombinant Jug r1 in order to evaluate their role in atopic diseases.

Walnut allergy is actually considered an emerging problem for public health; in fact the walnut represents the second leading cause, after peanuts, of anaphylactic reactions to dried fruit (1, 2). Furthermore, walnut consumption has recently become an healthy dietary habit worldwide, due to its positive benefits in reducing cholesterol levels and oxidative stress; this has resulted in an increase in individual consumption, global production and risk of developing sensitization and allergy (3). Walnut is also included as seed or oil in several cookery dishes and pastry products and may be present in trace amounts or hidden in many other foods, representing an healthy risk for sensitized and allergic individuals. Moreover, walnut hulls are also processed in dyes and inks industry, presenting elevated commercial interest. The walnut belongs to the botanical family of Junglandaceae, which includes many different species, but only two of these, the Junglans regia (common walnut) and Junglans nigra (black walnut),are actually consumed in the diet (4). The walnut allergy is reported in about 1-2% of children, with wide geographic variations and an increasing trend, and tends to be lifelong (5-7). In general, clinical manifestations of walnut allergy are frequently severe and systemic potentially life-threatening, leading to anaphylaxis both in the pediatric and adult populations (8). The type of allergen component sensitization may influence the severity of reactions. Referring only to common walnut, the molecular characterization of allergens includes prolamins (Jug r 1, Jug r 3), cupins (Jug r 2, Jug r 4) and profilins (Jug r 5), each with different clinical significance.

We retrospectively studied a group of children, sensitized to walnut, visited at the Service of Immuno-Allergy Pediatric Pneumology of IRCCS Policlinico San Matteo in Pavia. Patients were divided into two groups: children with reactions after the oral food challenge and children without reactions.

Skin tests (prick test and skin prick by prick) and the oral food challenge were performed, according to international guidelines (9). Serum levels of specific IgE were detected by immunofluorometric assay (ImmunoCAP; Thermofisher, Uppsala, Sweden) in

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patient peripheral blood samples. Specific IgE levels higher than 0.35 kU/L were considered positive (10). Total IgE was measured by a fluorescence immunoassay (ImmunoCAP; Thermofisher, Uppsala, Sweden) according to the manufacturer's instructions and the positivity depends on age. Jug r1 has been assayed by the semi-quantitative ISAC method (ImmunoCAP; Thermofisher, Uppsala, Sweden). Statistical analysis was performed using the statistical software package Medcalc 9 (Frank Schoonjans, BE). Medians and percentiles (25th and 75th, IQR) were used as descriptive statistics. The non-parametric Mann Whitney test was used to compare samples. In addition, a receiver operating characteristic (ROC) curve analysis was performed in order to determine a cut-off for specific IgE to Jug r1, that could optimize the sensitivity and the specificity of the test, to distinguish between responder and non-responder subjects. A p-value ≤ 0.05 was considered statistically significant.

Globally, 36 patients were visited (9 females, 25%, and 27 males, 75%; mean age 9.64 years - ds 3.45 years): 28 (77,78%) without reactions and 8 with a reaction like anaphylaxis (11,11%), angioedema (5,56%) or urticaria (5,56%). Clinical and immunological characteristics of the two group are reported in table 1. Allergen specific IgE levels to walnut and to Jug r1 were significantly higher in patients with a reaction.

ROC analysis has been performed for Jug r1, showing the best cut-off at 0,12 kU/L (area under the curve = 0,804; sensitivity = 87,5; specificity = 64,5), as shown in Fig. 1. The analysis showed an interesting result about the cut-off found, that is lower than the manufacturer's instructions. This is a useful result which could support clinicians to evaluate the risk of patients in doing oral food challenge; in this way they have a model to predict if the patients will have a reaction also with only one recombinant allergen. In fact, even if the oral food challenge actually represents the gold standard investigation for the objective diagnosis of IgE-mediated allergy, it must be performed in a specialist setting with emergency support immediately available, due to the risks of severe reactions, including anaphylaxis (11).

We performed this preliminary study considering only the walnut native allergen and the recombinant Jug r1; our aim is to continue this analysis, considering others walnut recombinants, to support the concept that traditional IgE assay and microarray should be integrated for an optimal diagnosis.

Table I. *Characteristics of the studied population.*

	Reaction: yes (N = 8)	**Reaction: no (N = 28)**	**p**
Age (yrs, mean(SD))	9.875 (4.15)	9.57 (3.305)	0.83*
Gender : male/female (No.of subjects (%))	5 (62.5%)/ 3 (37.5%)	22(78.57%)/6(21.43%)	1.00#
Total IgE (kU/l)	536 (86.125 - 1261)	1073.5 (276 - 1478)	0.27
Specific IgE for walnut (kU/L)°	37.65 (15.7 – 59.6)	1.30 (0.6075 - 2.77)	0.048
Specific IgE for Jug 1 (ISU-E)	3.985 (0.18 - 8.725)	0.025 (0 - 0.255)	0.008

All data are reported as median, with 25th and 75th percentiles in parentheses; P values refers to the Mann Whitney test, unless other specified.

** p value refers to the unpaired t-test*

 # p value refers to the Fisher exact test

° IgE values were available only for 2 patients with reaction and 11 without reaction.

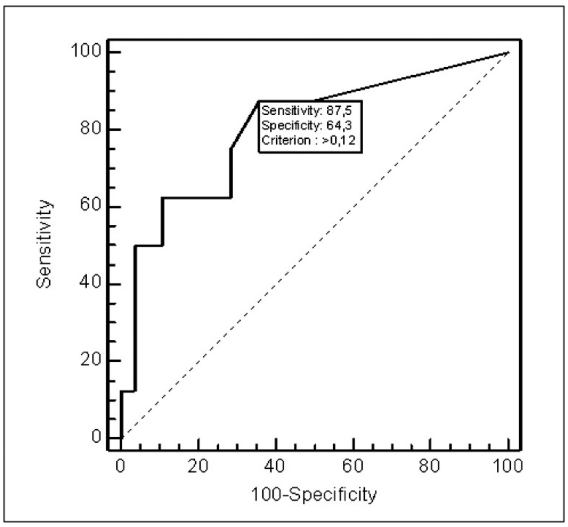

Fig. 1. *The Receiver operating characteristic analysis for Jug r1 showed a cut off of 0.12 kU/L.*

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