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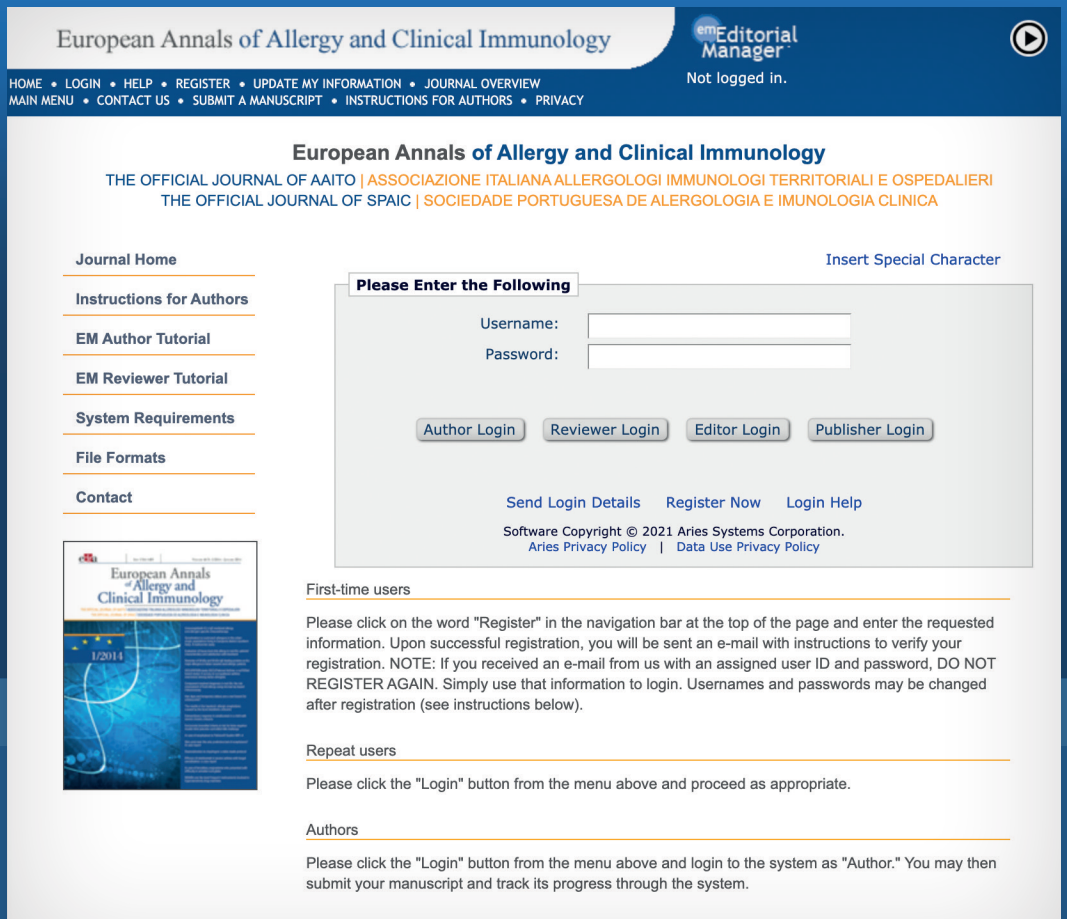
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Table of Contents

Review

Why is pet (cat/dog) allergen immunotherapy (AIT) such a controversial topic? Current perspectives and future directions 147
Gennaro Liccardi, Matteo Martini, Maria Beatrice Bilò, Lorenzo Cecchi, Manlio Milanese, Luisa Brussino, Enrico Motta, Paola Rogliani

Original articles

Vitulia questionnaire: a new instrument to evaluate quality of life in children aged 4-7 years with food allergy . . 154
Francesca Galletta, Salvatore Fasola, Stefania La Grutta, Giovanni Battista Pajno, Stefano Passanisi, Giuseppina Salzano, Andrea Barbalace, Ilenia Panasiti, Francesca Dimasi, Lucia Caminiti

Immunological and molecular study in children with combined immunodeficiency 161
Shams Kholoussi, Abeer Ramadan, Naglaa Kholoussi, Engy A. Ashaat, Alaaeldin G. Fayez, Haiam Abdel Raouf, Iman Helwa, Nora N. Esmaiel, Raghda Ghorab, Assem M. Abo-Shanab

A real-life cohort of mepolizumab treatment in severe eosinophilic asthma. 169
Daniel Laorden, Irene Hernández, Javier Domínguez-Ortega, David Romero, Rodolfo Álvarez-Sala, Santiago Quirce

Position paper

IgE-mediated reactivity to non-specific lipid transfer protein (nsLTP): clinical implications and management − a consensus document of the Association of Italian Territorial and Hospital Allergists and Immunologists (AAIITO) 176
Riccardo Asero, Valerio Pravettoni, Danilo Villalta, Lorenzo Cecchi, Enrico Scala

Case report

Ultrafast regimen for Pru p3 sublingual immunotherapy (SLIT-Peach®) in patients with anaphylactic LTP-Syndrome 183
Maria Soledad Zamarro Parra, Yulia Petryk Petryk, Javier Carbonell Zamorano, Antonio Carbonell Martínez

Letter to the Editor

Clinical characterization of peach allergic patients and respective molecular sensitization profile with ALEX®2 macroarray 188
Maria Inês T. Silva, Rita Brás, Marisa Paulino, Fátima Cabral Duarte, Maria Conceição Pereira Santos, Célia Costa

REVIEW

Eur Ann Allergy Clin Immunol Vol 56, N.4, 147-153, 2024

Gennaro Liccardi[1], Matteo Martini[2,3], Maria Beatrice Bilò[2,3], Lorenzo Cecchi[4], Manlio Milanese[5], Luisa Brussino[6], Enrico Motta[7], Paola Rogliani[1,7]

Why is pet (cat/dog) allergen immunotherapy (AIT) such a controversial topic? Current perspectives and future directions

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Key words

Allergic rhinitis; bronchial asthma; cat allergen; dog allergen; immunotherapy.

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Impact statement

Further studies are urgent both as regards the standardization of pet allergens and the improvement of the efficacy of the various modalities of the AIT and of other immunological approaches.

Summary

Dogs and cats are the most common pets worldwide. In Italy, the prevalence of allergic sensitization to cats and dogs is 16% and 9%, respectively. The limited standardization of allergenic extracts, especially for dogs, emphasizes the importance of component resolved diagnosis (CRD) for accurate diagnosis and subsequent prescription of allergen immunotherapy (AIT). However, this low standardization is the main factor contributing to the unsatisfactory clinical efficacy of traditional AIT, AIT with modified allergens, and intralymphatic allergen-specific immunotherapy (ILAIT). Emerging immunological approaches, particularly for controlling the primary cat allergen, show promise but are hindered by high costs (e.g., use of anti-Fel d 1 monoclonal antibodies in humans) or by exclusively targeting Fel d 1 produced by one's own animal (e.g., immunizing cats to induce neutralizing antibodies against Fel d 1 or including an egg product with anti Fel d 1 IgY antibodies in feline diet). Further studies are imperative for standardizing pet allergens, enhancing the efficacy of various AIT modalities, and exploring other immunological approaches, to optimize the relationship between pets and their owners and prevent distressing "forced removals".

Background and general aspects

Dogs and cats are the most prevalent pets globally. Recent data in Italy indicates that 44.7% of families own a dog, while 35.4% have a cat at home (Eurispes 2022) (1). Beyond the emotional connection, common pets drive substantial economic activities, including breeding, veterinary services, and new pet-related professions (*e.g.*, pet sitters, groomers, pet shop workers, mil-

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itary units) (2) and pet product industries (*e.g.*, pet-food, accessories).

The recent Italian Multicenter Study by Liccardi *et al.* (3) showed that allergic sensitization frequency to cats and dogs were 16% and 9% respectively. In Northern Europe and the US, higher pet ownership leads to much higher sensitization frequencies (up to 50%) (4). Conversely, China exhibits sensitization frequencies similar to Italy (14.9% for dogs and 9.3% for cats) (5). At present, both in dogs and in cats eight main allergens characterized by different molecular weights, biochemical properties, and biological functions have been identified (6). Notably, the primary cat allergen (Fel d 1) is responsible for over 90% of allergic sensitizations to cats, while dogs can have various allergens involved, such as Can f 1, Can f 2, and Can f 5 (7, 8). The distinct roles of common pet allergens in inducing sensitization underlie challenges in standardizing allergenic extracts and impact the clinical efficacy of allergen immunotherapy (AIT). Notably, the recently identified dog prostatic allergen (Can f 5) has become the most prevalent dog allergen in Italy (9) and globally (7, 8). Furthermore, according to a previous Italian multicenter study by Liccardi *et al.* (10), it has been showed that owning a male dog significantly increases the risk of mono-sensitization to Can f 5 compared to those exposed to female dogs or without direct contact with either gender. Polysensitization to dog and cat allergen components is associated with a high likelihood of allergic symptoms during exposure (11). Diagnosing dog allergies is particularly challenging due to difficulties in obtaining diagnostically accurate extracts with well-defined allergen compositions (12). The production of allergens (Can f 1-6) varies between dog breeds and anatomical sites, as well as between individual dogs of the same breed, introducing variability in natural extracts in terms of source, sampling, processing, and ultimately standardization and minimum allergen levels for accurate diagnosis and treatment (12).

Importance of molecular diagnosis (Component Resolved Diagnosis - CRD)

The limited standardization of allergenic extracts, particularly for dogs, has underscored the importance of molecular diagnostics (Component Resolved Diagnosis, CRD). It has been previously proposed that CRD is valuable in predicting the risk of sensitization to less common mammals, such as hamsters, rabbits, and horses, in individuals already sensitized to dogs or cats (13). Pre-existing sensitization to cats poses a lower risk due to the characteristics of its allergens, while sensitization risk is higher in individuals sensitized to dogs, attributed to cross-reactive allergens like lipocalins (13). Given the growing role of the dog-prostatic allergen (Can f 5) in sensitization compared to the well-known Can f 1 and Can f 2, we advocate for CRD prior to AIT prescription (14). If CRD reveals mono-sensitization to Can f 5, a common occurrence in dog-sensitized individuals (9, 10), a dog AIT containing a mix of allergens may have limited clinical efficacy (14). The EAACI Molecular Allergology User's Guide 2.0 (15) has emphasized the importance of IgE anti Fel d 1 for identifying primary sensitization in cat allergy cases, while IgE anti Can f 1, Can f 2, and Can f 5 indicate primary sensitization in dog allergy cases. The presence of these allergens is considered crucial for an effective therapeutic response during AIT. Schoos *et al.* (16) also provides similar guidance on primary sensitization to pet allergens, recommending nasal challenge tests to refine the pet AIT, in cases of uncertain results. It is imperative that such tests are conducted in controlled medical environments to address the potential occurrence of bronchial obstruction, which can be common in asthmatic subjects (17).

Traditional Allergen Immunotherapy (AIT) and novel modalities of immunologic approach to cat/dog allergic sensitization

Traditional Allergen Immunotherapy (AIT)

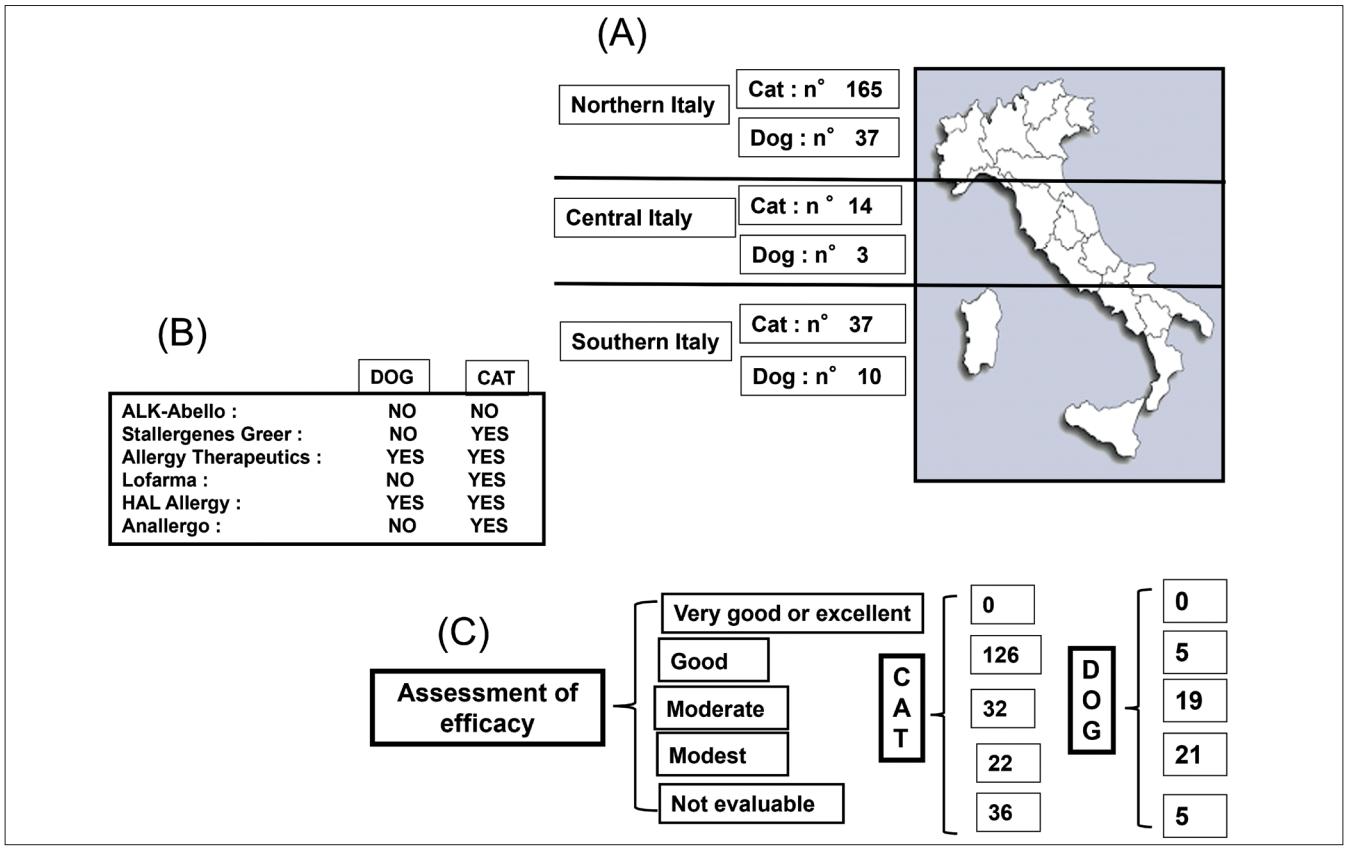
Data on cat/dog AIT in Italy

To provide current insights into subcutaneous (SCIT) or sublingual (SLIT) AIT for dog/cat allergens in Italy, we conducted an online survey encompassing both manufacturing companies and allergy centers evenly distributed across the country. We inquired with the manufacturing companies about the inclusion of AIT extracts for dogs/cats in their price lists, and gathered information from Italian allergists regarding the number of prescriptions issued in the last decade, as well as the clinical efficacy in managing respiratory symptoms. Six manufacturing companies and 30 allergy specialists from all Italian regions participated in the online survey. The results are summarized in **figure 1**. As shown in **figure 1B**, one company does not manufacture allergenic extracts for cat/dog AIT. Only 2 out of six produce extracts for dog AIT, while 5 out of six offer extracts for cat AIT in their catalogues. Prescription trends over the last decade for pet AIT are highlighted in **figure 1A**; they are notably higher for cat sensitization compared to dog sensitization, particularly in northern Italy as opposed to central and southern regions (likely influenced by reimbursement policies regarding AIT costs). Finally, **figure 2C** illustrates that, in numerical terms, clinical efficacy is reported as more favorable in patients receiving cat allergenic extracts than those receiving dog allergenic extracts.

Actual aspects of cat/dog AIT

While AIT for dog/cat epithelia has demonstrated positive outcomes in atopic dermatitis treatment (18), its primary application is in respiratory allergy. AIT can be a potential add-on treatment option when conventional management proves infeasible or ineffective, and when there is specific IgE sensitization

Figure 1 *- An overview on the state of allergen immunotherapy for cat/dog in Italy in terms of prescription* ***(A)****, manufacturers* ***(B)****, and reported efficacy* ***(C)****.*



combined with a clear correlation between clinical symptoms and exposure to that particular allergen.

A pediatric-oriented EAACI Position Paper (19) and a Consensus Document on pet allergy (20) comprehensively reviewed the available literature spanning from the early 1980s to the middle of the second decade of the 2000s, affirming the actual clinical significance of AIT for cat/dog allergens. Alvaro-Lozano *et al*. (19) noted limited high-quality evidence supporting AIT for furry animals in children, with insufficient data on cost-effectiveness. Evidence for AIT in cat allergy is similarly limited, while data for its effectiveness in dog allergy is notably absent, likely due to the lack of standardized extracts and variability in sensitization profiles.

Dàvila *et al*. (20) reported modest results concerning the clinical efficacy of AIT with dog extracts due to factors like the use of low-quality extracts, variations in allergenicity, and the complexity of the allergenic profile of dogs. In contrast, AIT with cat extracts showed both clinical and laboratory improvements. However, while there are double-blind placebo-controlled randomized clinical trials (21, 22), not all have optimal designs, and the patient cohorts tend to be limited. It's worth noting that each AIT product is distinct, making it unlikely that results achieved with one product can be generalized to others in the market with the same allergen.

The reduction of wheal area observed through skin prick tests (SPT) towards the allergen of AIT is a common finding after a suitable treatment period. In a preliminary report, it has been demonstrated that SLIT for dog allergen, after a year of treatment, not only induced positive clinical responses but also significantly reduced the areas of wheals assessed by SPT, not just against dog allergens, but also against those of other mammals like rabbit, horse, mouse, rat, hamster, and cow (23).

More recently, Uriarte and Sastre (24) investigated the effects of AIT for dogs and cats using an ultrarush (4 hours) SCIT schedule administered via subcutaneous infusion pump in patients with rhinitis and/or asthma. While no severe adverse reactions (ARs) were recorded, a greater number were noted compared to standard protocols, although this number was similar to that of longer rush protocols when patients were premedicated with antihistamine (25). The use of an infusion pump reduced the

number of injections and could be applied when indicated in real-life settings under appropriate medical supervision.
Subsequently, the same authors explored the use of high-dose SCIT in patients with allergic rhinitis and asthma caused by exposure to cat and dog dander using real-life clinical practice extracts (Alutard SQ, Alk-Abelló). Allergenic extracts were administered via an infusion pump over 3 sessions as part of a rush protocol (24), followed by monthly administration over 12 months, at the following concentrations of major allergens: Fel d 1, 15 μg/mL; Can f 1, 3.21 μg/mL; Can f 5, 0.72 μg/mL (26). A significant improvement was observed in FEV1, symptoms of rhinitis and asthma, Quality of Life (QoL), use of medication, Visual Analog Scale (VAS) score, and Asthma Control Test score at 6 months and continued at 12 months. Clinical improvement with cat extract was significantly better than with dog extract.
The safety profile of the rush up-dosing and maintenance phases was generally good, as reported in previous studies (27).
Finally, Uriarte *et al.* (28) conducted a real-life study to assess clinical efficacy in terms of symptoms, QoL, asthma control, and pulmonary function in patients undergoing SCIT with ultrarush up-dosing using the same dog and cat extracts as in their previous studies (26). They compared the results and also evaluated the immunologic changes caused by cat/dog specific AIT. A significant improvement was observed in rhinitis and asthma symptoms and in QoL, use of medication, VAS, and ACT at 1 month; these improvements persisted at month 6. Cat-allergic patients had a better response to SCIT than dog-allergic patients, as also seen in our previous study (26). This finding may be attributed to the higher concentration of Fel d 1 relative to Can f 1 or other dog allergens not contained in the extracts used.

Intralymphatic allergen-specific immunotherapy (ILAIT)

Intralymphatic immunotherapy (ILAIT) administers allergen extract directly to lymph nodes to induce rapid and effective immunological tolerance. Three injections are provided at 12-week intervals, resulting in fewer injections, a shorter treatment duration, a reduction of expense and noncompliance with therapy (29). However, Park *et al.* (30) examined therapeutic efficacy and safety of ILAIT with L-tyrosine-adsorbed extracts of various allergens including those of cat/dog in patients with allergic rhinitis. After 4 months of treatment, ILAIT with L-tyrosine-adsorbed allergen extracts does not induce a significant therapeutic efficacy in allergic rhinitis but can provoke moderate-to-severe systemic reactions and cause pain at the injection site.

AIT with modified allergenic extracts

Since increasing the concentration of allergens during standard AIT increases the risk of possible adverse reactions, various strategies have been adopted, such as physical or chemical modifications of allergenic materials which allow the administration of larger amounts, minimizing the risk of adverse reactions.
Sola *et al.* (31) developed a new allergoid cat dander extract (ACD) (Probelte Pharma S.L.U., Spain.) from a native cat dander extract (NCD) by modification with glutaraldehyde, and the optimal process control was determined by SDS-PAGE, DOT BLOT and determination of free amine groups. The ACD showed a significant loss of allergenicity compared to NCD obtaining a good safety profile, while maintaining the IgG-binding capacity.
Calzada *et al.* (32) investigated a new dog dander allergoid (R&D Allergy & Immunology Unit, LETI Pharma, Madrid, Spain) containing Can f 1 and Can f 5, it exhibited a low capacity to bind IgE and to activate basophils in dog allergic patients. Furthermore, it showed potent activation of Th1 mediators and induction of tolerance through Treg activation. This allergoid could offer a safer profile than the native extract and could be an effective immunotherapy treatment for dog allergic patients.

Novel modalities of immunologic approach to cat/dog allergic sensitization

Use of anti-Fel d 1 monoclonal antibodies in humans
AIT has been utilized for over a century, yet the prevailing protective mechanism remains poorly understood. One consistent observation is that increased allergen-specific IgG can competitively block allergen binding to IgE (33). Orengo *et al.* (34) investigated the contribution of allergen-specific blocking IgG as the protective mechanism of AIT using pre-selected monoclonal anti-allergen-blocking antibodies to suppress allergic symptoms. To explore this fundamental point, they generated two fully human IgG4 antibodies, REGN1908 and REGN1909, specific for Fel d 1, the major cat allergen. They reported that recombinant, allergen-specific blocking IgG antibodies perform comparably to allergen-specific IgG isolated from patients who successfully completed AIT. This effect translates to a rapid and sustained reduction in clinical symptoms in patients with cat allergy thereby offering evidence that allergen-specific blocking IgG play an important role in the protective mechanism of SCIT and may be a potential new and more rapid treatment approach for allergies (34).
Shamje *et al.* (35) showed that a single subcutaneous prophylactic dose of a combination of two anti-Fel d 1 (*Felis domesticus* allergen 1) monoclonal antibodies (REGN1908-1909) reduced nasal symptoms in cat-allergic patients challenged with cat allergen by suppressing FceRI-, FceRII-, and Th2-mediated allergic responses. Finally, the efficacy of these therapies have been demonstrated also in asthmatic patients with cat allergy. It has been suggested that passive administration of neutralizing mAbs targeting the predominant cat allergen Fel d 1 alone, but not Fel d 2, 4, 7, or 8, can prevent cat allergen-triggered asth-

ma responses and may inhibit type 1 immediate IgE-mediated responses (36). Notably, REGN1908/1909 was generally well tolerated, with profound and rapid effects that can last for at least 85 days (36).

Immunization of cats to induce neutralizing antibodies against Fel d 1

Thoms *et al*. (37) within the "One Health" (http://www.onehealthinitiative.com) paradigm, propose to reduce or prevent cat allergy in human subjects by immunizing cats against their own allergen. The rationale of this method is based on the fact that the major cat allergen (Fel d 1) is recognized by over 90% of cat allergic patients (38).

The authors conjugate vaccine consisting of recombinant Fel d 1 and a virus-like particle derived from the cucumber mosaic virus containing the tetanus toxin-derived universal T-cell epitope tt830-843 (CuMVTT) was used to immunize cats. Vaccinating cats against Fel d 1 induces specific antibodies capable of neutralizing the molecule in situ (*i.e*., in saliva or tears), making cats less allergenic to Fel d 1-sensitive human subjects (37). The same authors demonstrate that allergic symptoms of cat allergic owners were alleviated after the immunization of their cats (39). As a result of the owner being less burdened by their allergy, the QoL of their cat may be improved. The ability of allergic cat owners to better tolerate and increase the duration of their interactions with their pet can benefit the animal through better training and socialization and awareness of the animals' overall health. It is also important to note that, from a veterinary perspective, the overall health status of the cats was not compromised by the induction of auto-antibodies against Fel d 1 (39).

Feline diet with an egg product containing anti Fel d 1 IgY antibodies

Satyaraj *et al*. (40) demonstrated that it is feasible to reduce the immunologically active Fel d1 allergen from cats by incorporating anti-Fel d1 polyclonal IgY from chicken eggs into their diet. In fact, it has been demonstrated the efficacy of a rabbit polyclonal and an allergen-specific chicken IgY to bind to Fel d1 in cat saliva and block Fel d1-IgE binding and IgE-mediated basophil degranulation. These Fel d1 blocking antibodies present a novel approach for neutralizing cat allergens and potentially offer a safe and noninvasive method to reduce cat allergenicity (41). The anti-Fel d 1 IgY is safe for cats, based on a comprehensive safety study that fed an egg product ingredient with multiple levels of anti-Fel d 1 IgY, including levels much higher than those used in efficacy studies (42). In addition, based on the principle of allergen load reduction, complete elimination of Fel d 1 production is not necessary, as the approach described does not neutralize 100% of the cat's Fel d 1. Essentially, it transforms moderate and high Fel d 1-producing cats to the equivalent of low or moderate producers without altering the cat's overall production of the allergen (43).

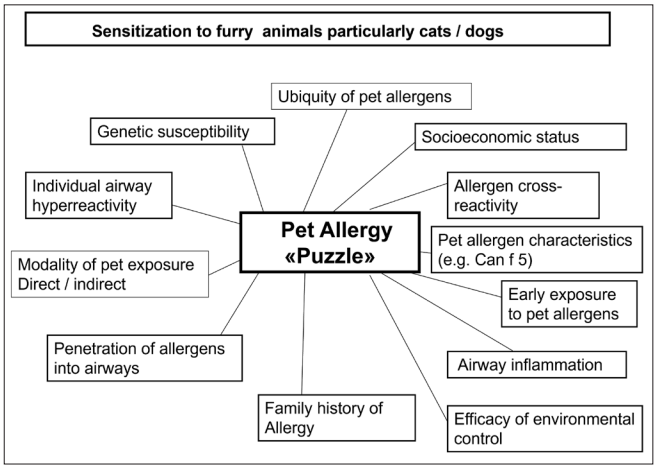
However, it is important to underline that the benefits for cat allergic patients, deriving from the use of monoclonal or IgY antibodies against Fel d 1, strictly concern the cat owned and in contact with the subject. Obviously, these benefits do not extend to other cats the patient may encounter, nor do they address "passive exposure" to cat allergens through various carriers such as clothing, hair, *etc*. (44, 45).

Concluding remarks

As shown in **figure 2** allergic sensitization to common pets, particularly dogs and cats, is a complex puzzle influenced by many different factors related to individuals, living environments, modes of exposure, and the animals themselves. The limited standardization of allergenic materials, especially for dogs, remains the primary factor contributing to the unsatisfactory clinical efficacy of traditional AIT, AIT with modified allergens, as well as ILAIT. While new immunological approaches, particularly for controlling the primary cat allergen such as the use of anti-Fel d 1 monoclonal antibodies in humans, immunization of cats to induce neutralizing antibodies against Fel d 1 and feline diet with an egg product containing anti-Fel d 1 IgY antibodies show great promise, they have significant costs (*e.g*., monoclonal antibodies) or the limitation of exclusively targeting one's own animal's Fel d 1.

The need for further studies is evident, encompassing both the standardization of pet allergens and the enhancement of the efficacy of various AIT modalities. This is crucial to optimize the relationship between animals and their owners and prevent the distressing necessity of "forced removals".

Figure 2 *- The "puzzle" of allergic sensitization to common pet allergens.*



Previous presentations

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Contributions

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Conflict of interests

The authors declare that they have no conflict of interests.

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Vitulia questionnaire: a new instrument to evaluate quality of life in children aged 4-7 years with food allergy

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Key words

Food allergy; health-related quality of life; questionnaire; children.

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Impact statement

The assessment of health-related quality of life in children with food allergy represents an important aspect in the management of this disease in every age group. Therefore, a new self-administered questionnaire among children aged 4-7 years could be a further useful tool to evaluate health-related quality of life.

Summary

Background. *Food allergy (FA) negatively affects health-related quality of life (HR-QoL) of children and caregivers. To date, no questionnaire self-compiling assessing the HR-QoL in pre-school children with FA is available. The aim of this study is to develop and validate a self-administered, rapid and easy questionnaire to evaluate the HR-QoL in children ≥ 7 years with IgE-mediated FA.* ***Methods.*** *A two-center prospective study was conducted including children aged 4-7 years with IgE-mediated FA. The Vitulia questionnaire was administered to study participants at the baseline (T0) and after one month (T1). To assess the feasibility and reliability, the Vitulia questionnaire was compared with other two pre-existing questionnaires: FAQLQ-PF and the KiddyKindl, which were also tested at both T0 and T1. The validation phase aimed to assess the following psychometric properties: convergent validity, internal consistency, discriminant validity and sensitivity to change.* ***Results.*** *One hundred patients (62% male, mean age 5.4 ± 1.2 years) were enrolled. The Vitulia questionnaire showed a good internal consistency along with an excellent reliability and repeatability of the measure. Another noteworthy feature of the questionnaire was its discriminant validity as demonstrated by the ability to provide different scores in subgroups, which have differences in terms of quality of life. On the other hand, the questionnaire seems not be sensitive to changes in health status over time.* ***Conclusions.*** *The Vitulia questionnaire represents a valid tool, quick and easy to interpret, which can be used to assess the quality of life in preschool children with IgE-mediated FA.*

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Introduction

Food allergy (FA) represents an emerging public health issue, with an increasing incidence in the last years (1). FA is the most important cause of life-threatening hypersensitivity reactions and negatively affects the health-related quality of life (HR-QoL) of patients and their caregivers, determining anxiety, fear of accidental exposures and social isolation (2).

The evaluation of HR-QoL in patients with FA is paramount for obtaining the best clinical control in the management of this disease (2). The assessment is performed through the administration of a standardized questionnaire, the Food Allergy Quality of Life Questionnaire (FAQLQ), submitted directly to patients aged ≥ 8 years or their parents if patients are younger than 8 years (FAQLQ-parent form). In the latter case, different versions are available according to the age of children (3-6). The most recent Food Allergy Quality of Life Questionnaire-parent form10 (FAQLQ-PF10) was also found to be a validate tool, with an excellent reliability in patients with FA (7).

However, in order to better evaluate the burden of FA, it would be important to receive a feedback directly from the child, even in the preschool age.

To the best of our knowledge, no questionnaire self-compiling assessing the HR-QoL in pre-school children with FA is available thus far. The aim of the study was to develop and validate a self-administered, rapid and easy questionnaire to evaluate the HR-QoL in children younger than 7 years affected by IgE-mediated FA.

Materials and methods

A multicenter prospective study was conducted between March 2019 and March 2022. The study population included children aged 4-7 years with IgE-mediated FA from at least one year and followed at the Pediatric Allergology Unit, University Hospital "G. Martino" of Messina or at the Institute of Translational Pharmacology, National Research Council of Italy of Palermo. Non-IgE mediated FA and poor compliance were considered as exclusion criteria. The study was approved by the Ethics committee of the University of Messina (n° 27/19). Parents of each patient provided their informed consent. Anamnestic and clinical data were collected. Skin prick test, prick by prick test and specific IgE dosage were performed. The diagnosis of IgE-mediated FA was confirmed through an oral food challenge. The Vitulia questionnaire was developed by two Pediatric allergy Centers, the University Hospital "G. Martino" of Messina and National Research Council of Italy of Palermo. The name comes from the imagination of the creator of the project (GBP). The Vitulia questionnaire was established based on current best practice for developing and validating scales for health, social, and behavioral research (8). This instrument was first tested on a small sample of the target population to assess its feasibility and usability. The selected sample consisted of 5 Italian-speaking patients with food allergy and their parents. Two medical doctors with expertise in psychology supervised the test and investigated on the eventual presence of any type of issues related to the questionnaire (*e.g.*, clarity, culturally inappropriate expressions or difficulties in understanding the questions). The debriefing interviews included paraphrasing each questionnaire item and assessing the clarity of the terms used and the overall understanding of the questions. Then, a group of medical doctors and psychologists discussed the feedback of the tested sample. Patients' parents had education levels ranging from middle school education to college education. Patients and parents reported that questions were simple and easy to understand, and no changes were suggested. Therefore, a final spelling, grammar and formatting check was performed leading to the final approval of the first Italian version of the questionnaire. In a second phase, the questionnaire was translated into English to make it understandable and usable in other countries worldwide. The translation was carried out by two native English speakers who have not a medical background. The translations were done independently, and the translators were not allowed to contact each other during the translation process. A panel of doctors compared the two versions translated into Italian which were identical for 100% of the content. This questionnaire consists of 10 items exploring several aspects of HR-QoL at T0 and any changes at T1. The questionnaire was printed and directly administered to the child. If the child had not yet acquired reading skills, the operator read the questions, and the child answered by indicating the corresponding emoticon/number (**figure 1**). The total score was expressed with a scale from 1 (best quality of life) to 3 (worst quality of life).

This questionnaire was administered to all the patients recruited in the study at the baseline (T0) and after one month (T1), directly at home, via mail, or during a follow-up visit.

To assess the feasibility and reliability of the Vitulia questionnaire, we compared our instrument with other two pre-existing questionnaires: the before mentioned FAQLQ-PF (4) and the KiddyKindl (9). FAQLQ-PF questionnaire involves 30 items, and the total score is expressed with a scale from 0 (best quality of life) to 6 (worst quality of life) (4). KiddyKindl is a generic instrument for assessing HR-QoL in children aged 4-6 years (9). It involves 12 items, and the total score is expressed with a scale from 0 (worst quality of life) to 100 (best quality of life) (9). Both the FAQLQ-PF and KiddyKindl questionnaires were administered at baseline and after one month. At T1, all the patients also indicated changes in their health status through a global scale of change (GSC), with a

Figure 1 *- Vitulia questionnaire.*

VITULIA QUESTIONNAIRE

The following questions regard your food allergy.
You can ask your parents for help, but they can't tell you the answers.
Write "X" in the box of your chosen answer.

You can choose from these answers:


Very discomfort

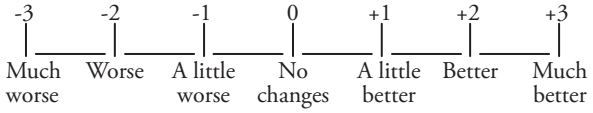

Little discomfort


No discomfort

How much it bothers you ...			
1. Have a food allergy			
2. Be always careful what you eat			
3. Eat only certain foods			
4. Don't eat the same things as your classmates/friends			
5. Not knowing the taste of some foods			
6. See classmates/friends eat all foods			
7. Feel bad if you ate foods you can't			
8. Don't try new foods			
9. Eat outside home (parties of friends, school trips…)			
10. Do not eat the food you are allergic to			

Emotions: 1,2,4,6,7
Restrictions: 3,5,8,9,10

How is your allergy since your last visit?



score from 0 (no change) to ± 3 (much better/worse). Clinically relevant improvement was defined as GSC > 1.
The aim of the validation phase was to assess the following psychometric properties:

1. Convergent validity: both at T0 and at T1, the correlation between the Vitulia score and the KiddyKindl and FAQLQ-PF scores was evaluated through the Spearman's rho correlation coefficient, considering as acceptable coefficients of about 0.5.
2. Internal consistency: both at T0 and at T1, the consistency of the questions was evaluated through the Cronbach's alpha coefficient. Values greater than 0.7 were considered acceptable.
3. Reliability was evaluated through the intra-cluster correlation coefficient (ICC) calculated on the pairs of repeated measures (T0 and T1) in children with GSC = 0. The interpretation of the ICC was: low (0 to 0.20), acceptable (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80), excellent (> 0.80).
4. Discriminant validity was evaluated by comparing questionnaire scores in children forced to avoid 0-2 foods (average low expected score), and in those forced to avoid more than 2 foods (average high expected score).
5. Sensitivity to change (GSC > 1) was assessed using the Wilcoxon rank test for paired data, applied to the mean change in scores between T0 and T1. Statistical significance was defined as a P-value < 0.05.
6. Floor/ceiling effects (*i.e.*, missing extremes in the lower/upper end of the scale) were considered present if over 15% of respondents achieved the lowest (*i.e.*, 1) or highest (*i.e.*, 3) possible score.

Results

One hundred patients (62% male, mean age 5.4 ± 1.2 years) were enrolled. Children characteristics are shown in **table I**.
No correlation was found between Vitulia questionnaire and KiddyKindl both at T0 and T1 (p = 0.305 and p = 0.106 respectively). On the contrary, acceptable correlations were found between the Vitulia questionnaire and the FAQLQ-PF (0.40 at T0 and 0.56 at T1, p < 0.001) (**table II**). Cronbach's alpha coefficient for the Vitulia questionnaire total score was 0.88 at T0 and 0.89 at T1. For the "emotions" subdimension (items 1, 2, 4, 6, 7) the coefficient was 0.77 at T0 and 0.81 at T1. For the "limitations" subdimension (items 3, 5, 8, 9, 10) the coefficient was 0.80 at T0 and 0.81 at T1 (**table II**). The ICC for the pair of repeated measures (T0 and T1) of the Vitulia questionnaire in the 23 children with GSC = 0 was 0.84, with a 95% confidence interval of (0.66-0.93) (**table II**).
At both visits, the average score of the new questionnaire was significantly higher in the group of children forced to avoid more than 2 foods than in those avoiding £ 2 foods (2.3 ± 0.5 *vs* 2.0 ± 0.5 and p = 0.021 at T0; 2.3 ± 0.5 *vs* 2.0 ± 0.6 and p = 0.002 at T1) (**figure 2**). Among the 46 children who reported clinically relevant improvements (GSC > 1), the mean change in scores between T0 and T1 was not statistically significant (-0.05, p = 0.364). The lowest possible score (*i.e.*, 1) was achieved by 2% of respondents at T0 and 3% at T1, while the highest possible score (*i.e.*, 3) was achieved by 10% both at T0 and at T1 (no floor/ceiling effects).

***Table I** - Characteristics of children at baseline (T0).*

Age, years, no. (%)	
4	35 (35%)
5	18 (18%)
6	16 (16%)
7	31 (31%)
Gender, no. (%)	
Female	39 (39%)
Male	61 (61%)
Parent answering, no. (%)	
Mother	71 (71%)
Father	20 (20%)
Both	9 (9%)
KiddyKindl score, mean (SD)	78.3 (12.4)
FAQLQ score, mean (SD)	2.4 (1.5)
Vitulia score, mean (SD)	2.2 (0.5)
Avoided foods, no. (%)	
0-2	37 (37%)
>2	63 (63%)
Global Rating of Change (GRC, at T1)	
-3	1 (1%)
-2	0 (0%)
-1	2 (2%)
0	23 (23%)
+1	28 (28%)
+2	20 (20%)
+3	26 (26%)

SD: standard deviation.

Discussion and conclusions

The Vitulia questionnaire showed a good internal consistency along with an excellent reliability and repeatability of the measure. Another noteworthy feature of the questionnaire was its discriminant validity as demonstrated by the ability to pro-

Table II *- Convergent validity, internal consistency, and reliability of Vitulia: indicators and results.*

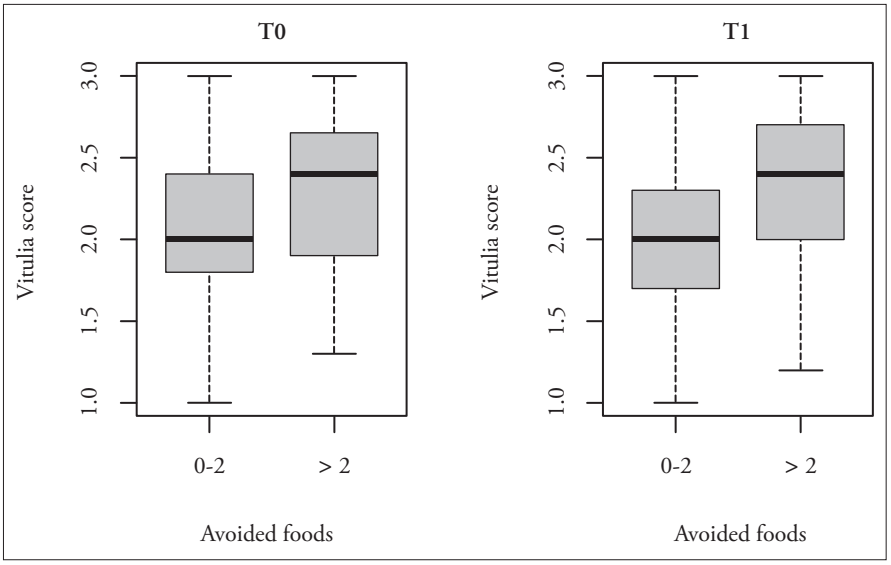
Psychometric property	Indicator	Results
Convergent validity	Spearman's rho correlation coefficient of Vitulia and other instruments	KiddyKindl, T0: rho = -0.10 (p = 0.305) KiddyKindl, T1: rho = -0.16 (p = 0.106) FAQLQ, T0: rho = 0.40 (p < 0.001) FAQLQ, T1: rho = 0.56 (p < 0.001)
Internal consistency	Cronbach's alpha correlation coefficient of Vitulia	T0, total score: alpha = 0.88 T0, emotions: alpha = 0.77 T0, limitations: alpha = 0.80 T1, total score: alpha = 0.89 T1, emotions: alpha = 0.81 T1, limitations: alpha = 0.81
Reliability	Intra-cluster correlation coefficient (ICC) for Vitulia repeated measurements (T0-T1) in children with GRC = 0	ICC = 0.84 [0.66-0.93]

vide different scores in subgroups, which have differences in terms of quality of life. In developing the questionnaire, we deliberately emphasized clinical features that revealed the most important aspects according to the perception of children with food allergies. We therefore identified two subdimensions: "emotions" and "limitations," for which the Cronbach's coefficient resulted in > 0.70 both at T0 and T1.

Our results supports the need for a disease-specific HR-QoL questionnaire in food allergic preschool children. The content of the Vitulia questionnaire considers the most important issues that children with food allergy have to deal with in their daily life, at school, at home or outside, impairing their HR-QoL (10). As a result, these issues are likely to constitute significant focal points for interventions by healthcare providers, food manufacturers, and also caregivers (11).

The aim of these efforts is to enhance the quality of life for individuals with food allergies. For example items such as "*Don't eat the same things as your classmates/friends*" and "*See classmates/friends eat all foods*" have highlighted a real discomfort in children. This finding focuses the need to raise awareness of food

Figure 2 *- Discriminant validity of the Vitulia questionnaire: score distribution by number of avoided foods at baseline (T0, left panel) and after one month (T1, right panel).*



Boxplots represent the median (central line), 25th-75th percentiles (box), and min-max non-outlier values (whiskers).

allergy within the community. While there have been recent improvements in labeling legislation for food-allergic patients in Europe and the USA, there has also been an increase in the use of precautionary labeling (12). The impact of this trend on the quality of life is exemplified by the statement "*Be always careful what you eat*". This underscores the need to explore new therapeutic interventions that could enable children with food allergies to consume the foods they are allergic to, thereby potentially enhancing their quality of life (13).

The Vitulia questionnaire well discriminates between children who reported more than two food allergies and those who reported two or fewer. At both visits, the average score of the new questionnaire was significantly higher in the group of children forced to avoid more than 2 foods compared to those avoiding two or fewer. This finding also reflects a greater impact on the quality of life in children who experienced greater dietary restrictions, according to literature data (10).

Moreover, as we expected, significant correlations emerged between the Vitulia questionnaire and FAQLQ-PF, concerning similar relevant issues such as emotional impact and social restrictions. Unlike the FAQLQ-PF, Vitulia questionnaire investigates the most important clinical aspects related to quality of life in ten simple items and is directly administered to preschool children, who can complete it even without parental assistance. This represents a strength of our questionnaire, as children and parents differ in their opinions about quality of life. Parents are less capable of assessing symptom experiences and peer relationships from the children's perspective (14).

Nevertheless, no correlation was found with the questionnaire KiddyKindle, probably due to the generic investigation of the HR- QoL of children, unlike Vitulia, which is specific to food allergies.

Finally, our study presents some limitations. The validation process of the Vitulia questionnaire was conducted in Italian language, even though it was carefully translated into English. However, it is possible that cultural and linguistic differences may influence the questionnaire's ability to identify the most important aspects for children with food allergies (15). Moreover, among the 46 children who reported clinically relevant improvements (GSC > 1), the mean change in scores between T0 and T1 was not statistically significant, consequently the questionnaire seems not be sensitive to changes in health status over time. However, this could be due to the low reliability of using the GSC in the age group studied.

In conclusion, we presented the first self-administered disease specific HR-QoL questionnaire for children aged 4-7 years affected by food allergy. The analysis of our data suggests that Vitulia questionnaire represents a valid tool, quick and easy to interpret, which assess the most important challenges that children with food allergies encounter. The introduction of this new instrument in daily clinical practice would allow to conduct studies where assessing quality of life is the primary end-point. Examples include evaluating emerging therapies, such as oral immunotherapy and anti-IgE therapy, not only concerning the effectiveness and safety but also related to a significant reduction in the burden of the disease.

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None.

Contributions

GBP, SLG, LC: conceptualization. AB, IP, FD, FG: data curation. SF, SLG: formal analysis. FG, SF, SP: writing – original draft. GS, SP: supervision, writing – review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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Immunological and molecular study in children with combined immunodeficiency

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Impact statement
This study explores genetic defects in children with SCID, identifying mutations in the lymphoid-specific recombinase activating RAG1 and RAG2 genes, highlighting the importance of comprehensive genetic analysis for accurate diagnosis and treatment.

Summary
Background. *Severe combined immunodeficiency (SCID) is a form of immunodeficiencies (PID), caused by molecular defects. These defects can restrict the development and function of lymphocytes. Early diagnosis and treatment of SCID can lead to disease-free survival. This study aims to investigate some of the possible underlying genetic defects in a group of Egyptian infants and children with clinical and immunological profiles suggestive of SCID.* ***Methods.*** *This study included eighty patients who showed clinical warning signs of immunodeficiency. Subjects were thoroughly examined clinically. Laboratory evaluation included immunoglobulins serum levels and flow cytometric assessment of immune cells. This testing showed an altered immune profile in thirty patients. They had decreased T and/or B lymphocytes or natural killer cells. DNA extraction was done for those cases. The coding regions of the RAG1 gene and RAG2 gene was investigated for hot spot mutations by sequencing technique guided by the patient clinical evaluation, inheritance pattern, immunophenotyping by flow cytometric analysis of lymphocyte subsets, and serum immunoglobulins level detection.* ***Results.*** *The results showed novel and previously reported variants (mutation, polymorphism), they were found in 18 cases which include variants in the RAG1 gene (E880K, A960A, H249R, S913R, K820R, V782G), and variants in the RAG2 gene (P501T, L514M, rs10836573, cDNA.2129A>T).* ***Conclusions.*** *To evaluate SCID patients completely, mutation gene analysis is highly required and recommended.*

Introduction

Primary immunodeficiencies (PID) are a heterogeneous group of disorders with a broad range of clinical symptoms. Most of the primary immunodeficiency diseases are monogenic disorders and inherited as autosomal recessive forms. The prevalence of the various PIDs varies in different countries (1). In the Middle East and North Africa, the rate of registered patients ranges between 0.02 and 7.58 per 100,000 population with a total of 17,120 patients registered. The prevalence rate is 0.68 per 100,000 population in Egypt (2). Another 343 patients with monogenic diseases of immune dysregulation were registered in the same region where Egypt constituted 5% of patients (3). Severe combined immunodeficiency (SCID) is a very severe form of PID, and early diagnosis can be lifesaving (4). SCID syndromes occur

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more frequently in males; the prevalence of which are nearly 1:50,000 live births. This reflects the excessive representation of X-linked SCID. Despite this, when high consanguinity populations are studied, autosomal recessive SCID occurs at a higher frequency than previously reported (5). SCID occurs when molecular defects are encountered and affect lymphocyte development and function. These defects may also restrict the differentiation and proliferation of T cells, B cells as well as natural killer (NK) cells. Although mature B cells may be normally present, antibody production is markedly compromised due to a lack of T-cell help. Moreover, NK cells are also affected which renders the innate immunity to threats as well (6). At birth, SCID may not be explicit clinically. Thus, infants may present to medical clinics with complications of infections that are indistinguishable from repetitive childhood infections. This accordingly delays diagnosis in a significant manner. During the first year of life, these infants may die of infectious complications (7). Nevertheless, routine administration of live vaccines may put them at risk of developing "Vaccine Associated Paralytic Poliomyelitis" (VAPP) and BCGosis following OPV and BCG administration (8).

Early diagnosis of SCID is highly recommended. Starting treatment and avoiding infections and complications will reduce hospitalization costs and draw attention to early stem cell transplantation. This will lead to disease-free survival in immune-compromised infants. Testing immediately after birth can be done by sequencing DNA if the family mutation is known or by estimation of the numbers of T, B, and NK cells. On the routine evaluation of SCID patients, lymphopenia and altered absolute lymphocyte count (ALC) at birth help in pre-symptomatic detection and early medical interference. However, ALC may show normal in some cases as it is not as sensitive and specific compared to molecular testing (9). In SCID evaluation, mutation analysis is gaining more importance especially because the list of disease-associated genes is continuously increasing (10). Moreover, recognizing the disease-associated genes of this potentially life-threatening disease will have an intense influence on several disease aspects as the value of genetic counseling and prenatal diagnosis. In addition, prognosis and treatment modalities will remarkably advance (11).

Materials and methods

This study was approved by Medical Ethics Committee no (16/108). Informed consent from the parents or guardians of patients included in our study was obtained according to the rules of the Medical Ethics Committees in the National Research Centre (NRC). 80 patients were included in this study. They were subjected to clinical evaluation (demographic data – name, age, sex, social class; full history taking stressing on:

a. birth history – length of gestation, birth weight, perinatal and neonatal problems, such as jaundice, respiratory distress, or need for intensive care.
b. transfusions in the neonatal period
c. delayed detachment of the umbilical cord beyond 30 days
d. feeding history including food intolerance and duration of breastfeeding
e. any adverse effects from a vaccine particularly live virus vaccines
f. the presence of family members with similar diseases, recurrent infections, unexplained death, or autoimmune disease suggests the possibility of a genetic illness
g. the infection history – the age of onset, duration, frequency, sites, organisms, treatment, and response to therapy.
h. thorough clinical examination – any abnormal clinical finding elicited during the clinical examination was recorded as well as any of the ten warning signs of primary immunodeficiency in children (12)).

Laboratory analysis

Complete blood count

Total leucocytic count (TLC) and differential counts were recorded, and absolute lymphocyte number was calculated. Measurement of serum immunoglobulins (IgG, IgA, and IgM) were done by nephelometry using a binding site Kit (13). Measurement of peripheral blood lymphocyte subsets percentage including the T-cell subset (CD3, CD4, CD8), B-cell (CD19), and natural killer cell (CD 16) were done by flowcytometry (14).

After flow cytometry analysis, cases studied (n = 80) were categorized into 2 groups according to T lymphocytes % and/or B lymphocytes concerning their age range (15).

group I (n = 50) with normal T and B lymphocytes; group II (n = 30) with decreased T and/or B lymphocytes. This group was further subjected to molecular studies.

Molecular studies

Eleven sets of primers were used for the amplification of the whole *RAG1* gene and *RAG2* gene (**table I**).

The coding regions of the suggested genes were sequenced using ABI PRISM® 3130 Genetic Analyzer.

Three fragments were purified for each case, and then purified fragments were sequenced.

Mutational analysis

Mutational analysis of the suspected gene defect was performed guided by the patient clinical evaluation and flow-cytometric phenotype of the lymphocyte subset.

Table I *- The Sequence of PCR primers.*

Fragment	*Sequence (5'-3')		Primer
690 bp	CAAGCCAACCTTCGACATCT	F	RAG1 E2a
	GATATCGGCAAGAGGGACAA	R	
502 bp	CGCCAACTGCAGTAAGATACA	F	RAG1 E2b
	ACACGGACTTCACATCTCCACCT	R	
600 bp	CCACATCTCAAGTCACAAGGAA	F	RAG1 E2c
	TCCATGTCCATCAAAGCAGA	R	
819bp	GCCATCACAGGGAGACAGAT	F	RAG1 E2 d
	GGAACGCCAGACCTCATAAC	R	
594bp	GCACCGGCTATGATGAAAAA	F	RAG1 E2 e
	CACTCTTTAGCAGGGCATGA	R	
800	TCATGAGGATGAATGGCAAC	F	RAG1 E2 f
	TCCTGTTGCTTTTCGGAACT	R	
693	CCATAAACACTGTCAGAAGAGGAA	F	**RAG1**
	ACATCGATCCGGAAAACCTT	R	**Exon 1**
494	AGCGTGTGGGAGGACTTAAAA	F	RAG2
	AACCTTTTTGTTGTTCTTGCAAAC	R	**Exon 1**
497	GGAGGGAAAACACCAAACAA	F	RAG2
	TGGAGACAGAGATTCCTCCTG	R	**Exon 2a**
812	ATAATATCCGGCCTGCCAAC	F	RAG2
	GTAGGACTCTTTGGGGAGTGTG	R	**Exon 2b**
700	TCTACTGCTCTCATGGGGATG	F	RAG2
	GCCAACTGCCGAATTGTTAT	R	**Exon 2c**

*Primers are listed from 5' to 3' ends and the second of each pair is the reverse or antisense sequence. F: forward, R: reverse (14).

Statistical analysis

Statistically analyzed data was performed by using SPSS version 16.0 software. A nonparametric Mann-Whitney U test was used to compare concentrations of parameters between patients and healthy controls. Association between parameter expressions with age, gender, and total leucocytic count of patients was tested using Spearman rank correlation. Data were presented as mean ± SD. A P-value < 0.05 was deemed statistically significant.

The detected variants were subjected to bioinformatics analysis by a proper bioinformatics algorithm.

Results

This study included eighty patients who had warning signs of primary immunodeficiency. Patients were categorized into 2 groups according to the results of flow cytometric analysis into 2 groups: group I whose results revealed a normal count of T and B lymphocytes and group II which had decreased T and/or B lymphocytes. Group II was subjected to further molecular studies. **Table II** shows a comparison between both groups regarding age and gender.

Table II *- Age and gender for Group I and II.*

Parameter	Group I n = 50	Group II n = 30
Age range	10 days-3 years	7 days-2.5 years
Gender (%)		
M	25 (50%)	16 (53%)
F	25 (50 %)	14 (47%)
Consanguinity		
+ve	24 (48%)	17 (57%)
-ve	26 (52%)	13 (43%)

A statistically significant decrease in absolute lymphocyte count was shown in group II, the P-value was 0.0107 as compared to group I. Also, a statistically significant decrease in CD3%, CD19%, and CD8% was detected in group II as compared to group I, the P-value were 0.0001, 0.038, and 0.0022, respectively (**table III**).

A statistically significant decrease in both IgM and IgG in group II as compared to group I is detected, P-value were 0.0072 and 0.036, respectively (**table IV**).

The coding regions of genes (*RAG1* and *RAG2* genes) were investigated for hot spot mutations by sequencing technique guided by the patient clinical evaluation, inheritance pattern, flow-cytometric phenotype of lymphocyte subsets and serum immunoglobulin subtypes level detection.

Table III *- White blood cell characteristics and lymphocyte subsets by flowcytometry for Group I and II.*

Parameter	Group I, n = 50 Mean ± SD	Group II, n = 30 Mean ±SD	P-value
TLC10^3/mm^3	11.44 ± 1.46	11.31 ± 1.67	0.7289
Absolute lymphocyte count/mm^3	6.01 ± 1.61	4.44 ± 1.53	0.0107*
CD3%	52.34 ± 9.57	37.98 ± 16.26	0.0001*
CD19%	16.01 ± 6.08	12.33 ± 6.37	0.038*
CD16%	16.97 ± 5.91	19.47 ± 7.75	0.206
CD4%	27.45 ± 7.19	23.64 ± 10.01	0.1135
CD8%	22.33 ± 6.66	15.94 ± 8.1	0.0022*

*Significant difference at P-value < 0.05; TLC: total Leukocytic count.

Table IV *- Immunoglobulins levels in Group I and II.*

Parameter	Group I, n =50 Mean ± SD	Group II, n=30 Mean ± SD	P-value
IgA (g/L)	0.91 ± 0.62	0.66 ± 0.58	0.1591
IgM (g/L)	1.04 ± 0.54	0.72 ± 0.37	0.0072*
IgG (g/L)	11.46 ± 5.05	9.04 ± 2.92	0.036*

*Significant difference at P-value < 0.05; Ig: Immunoglobulin.

Successful PCR amplification of seven fragments in the *RAG1* gene (exon 1, exon 2a, exon 2b, exon 2c, exon 2d, exon 2e, exon 2f) and four fragments in the *RAG2* gene (exon 1, exon 2a, exon 2b, exon 2c) in affected cases (**table V**).

Our results showed novel and previously reported variants (mutation, polymorphism), they were found through 18 cases which include variants in *RAG1* gene (E880K, A960A, H249R, S913R, K820R, V782G), and variants in *RAG2* gene (P501T, L514M, rs10836573, cDNA.2129A>T).

The clinical manifestations of SCID patients was represented in (**table VI**); furthermore, in 18 cases diagnosed molecularly as SCID, 9 patients (50%) were of positive consanguineous parents.

Discussion and conclusions

This study included eighty patients who showed clinical warning signs of immunodeficiency. After flow-cytometric analysis,

Table V *- Different in silico prediction algorithms: SIFT, PolyPhen-2, and condel for the detected variants.*

DNA changes	AA changes*	Status	MT**/Grantham Matrix score (ref range; 0-215)	Splice sites modifications (wt:mu scores)***	SNP pred. score****		Condel
					SIFT	PolyPhen	
cDNA.2750G>A rs4151033	E880K	Reported	Disease-causing/56	Donor increased (wt: 0.47 / mu: 0.83)	0.04	0.991	0.793
cDNA.2992A>G rs1980131	A960A	Reported	polymorphism	None	-	-	-
cDNA.858A>G rs3740955	H249R	Reported	Polymorphism/29	None	0.38	0	-
cDNA.2851T>A RAG1	S913R	Novel	Disease-causing/110	Donors gained (0.44) Acc increased (wt: 0.21 / mu: 0.47)	0.03	0.999	
NM_001243785.1:c.1540T>A RAG2 Exon 2c	L514M	Novel	Polymorphism/15	Donor gained (0.61)	1	0.001	--
NM_001243786.1:c.1501C>A RAG2 Exon 2c rs781104028	P501T	reported	Disease-causing/38 moderate	Donor gained (0.37) Donor increased (wt: 0.47 / mu: 0.62)	0.03	0.073	--
cDNA.2129A>T RAG2	3'UTR	Novel	Predicted polymorphism	Donor marginally increased (wt: 0.8292 / mu: 0.8545)			
NM_000536.3 (RAG2):c.*328A>G rs10836573	-	reported	Polymorphism	-	-	-	-
NC_000011.9:g.36597313A>G cDNA.2571A>G rs2227973 RAG1	Lys820Arg K820R	Reported	Polymorphism/26	Donor gained (0.72)	0.13	0.006	
cDNA.2457T>G NM_000448.2:c.2345T>G RAG1	V782G	Novel	Disease-causing/109	-	0.01	0.994	

*AA changes: Amino Acid changes; **MT: Mutation Taster, this score is taken from an amino acid substitution matrix (Grantham Matrix) which considers the Physio-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid. Scores may range from 0.0 to 215; ***wt: Wild; mu: Mutant; ****SIFT: Amino acids with probabilities < 0.05 are predicted to be deleterious; PolyPhen-2: Polymorphism Phenotyping v2 for annotating coding nonsynonymous SNPs, variants scores between 0.0 to 0.15 are predicted to be benign. 0.15 to 1.0 are possibly damaging. 0.85 to 1.0 are more confidently predicted to be damaging; condel: CONsensus DELeteriousness score of missense mutations.

Table VI - *Clinical manifestations of SCID patients.*

No	Gender	AOO	AOD	Consanguinity	Main presenting features	Sibling death or abortion
1	M	3.5m	5 m	-ve	FTT, skin lesions, hypopigmented areas, pneumonia	His sister died at 3m by pneumonia
2	M	7 days	1 m	+ve	FTT, chest infection	Two sisters' death
3	Twin M/F	3m	4 m	-ve	FTT, pneumonia	First pregnancy
4	M	53 days	2 m	+ve	Pneumonia, chest infection, FTT	History of 4 neonatal death and IUFD
5	M	10 days	1m	-ve	FTT	History of abortion
6	F	1.5y	1.7 y	+ve	FTT, pneumonia	Patient was first child
7	F	1w	1 m	+ve	FTT, pneumonia	1 neonatal death
8	M	11days	1 m	+ve	RDS	2 neonatal deaths
9	M	5 days twin	1 m	-ve	Pneumonia	Abortion once
10	F	1.5y	2 y	+ve	FTT, pneumonia	1 neonatal death
11	F	8 ys	9 y	-ve	Severe pneumonia	First child
12	F	1.2 y	2 y	-ve	Severe pneumonia	1 neonatal death
13	F	19 days	1 m	-ve	Severe pneumonia	First child
14	F	8m	10 m	-ve	FTT	First child
15	M	1m	2 m	+ve	FTT, pneumonia	1 neonatal death
16	M	2m	4 m	-ve	Recurrent pneumonia	Abortion twice
17	M	1m	2 m	+ve	FTT, skin lesions, pneumonia	2 neonatal deaths
18	F	2w	1 m	+ve	FTT, pneumonia	Abortion once

AOO: age of onset; AOD: age of diagnosis; FTT: failure to thrive; RDS: respiratory distress syndrome; IUFD: intrauterine fetal death.

the cases studied (n = 80) were classified into two groups according to T lymphocytes and/or B lymphocytes percentages concerning their age range. Group I (n = 50) with normal T and B lymphocytes and group II (n = 30) with reduced T and/or B lymphocytes. Group II underwent further molecular studies that yielded 18 cases of SCID from 30 samples sequenced with identified variants (mutation or polymorphism) in the *RAG1* and *RAG2*.

T lymphocyte differentiation blockade is a main feature of SCID. Irregular development of lymphocyte lineages − whether B and/or natural killer cells − may inconsistently accompany the disease. Unless a hematopoietic stem cell transplant is offered; early death is usually inevitable (16). SCID includes heterogeneous cases, genotypically and phenotypically. The genetic origins of nearly 85% of the original immunologic defects have been lately clarified. The fact that diverse mutations in a single gene may cause different clinical conditions renders interpreting SCID pathogenesis a major obstacle. Moreover, similar clinical phenotypes can result from different gene mutations (17).

Severe combined-immune deficiency is characterized by severe and persistent infections from early life, which are due to profound impairment of both cellular and humoral immune function. Classically, the disease usually presents with an absence of both T and B cells. This is probably related to lymphocytic stem cell affection. Deficiencies in some enzymes as adenosine deaminase or purine nucleoside phosphorylase may lead to autosomal recessive forms of SCID. These types are either of yet unknown cause or due to IL-2 deficiency or related to the deficiency in major histocompatibility complex class II (bare lymphocyte syndrome) (18).

Accurate counseling and identification of the genetic defect are necessary for SCID to help parents to take rational decisions following pregnancies. Different ages of onset were revealed among patients in previous studies in addition to diverse molecular causes. However, this spots that in high-risk families: newborn screening is crucial (19). As to Meshaal *et al*. in 2015, *RAG* mutations were proved to be common among SCID cases in Egypt (20).

Early diagnosis is recommended in in PID patients order to decrease the risk of infectious and non-infectious conditions. Not only does this improve the disease outcome and quality of life, but also prevents serious complications (21). Aggressive respira-

tory and gastrointestinal tract infections are typical presenting symptoms that occur in their first year of life. Nevertheless, older children, up to 15 years, may present with milder symptoms. Initial evaluation of immunodeficiency cases after presenting to the clinic, including a few screening tests. Ranging from basic complete blood count (CBC), together with an immunologic profile including estimation of immunoglobulins and lymphocyte subsets (22).

Results of this study revealed a statistically significant decrease in IgM and IgG levels in group II as compared to group I; this agrees with Stepensky *et al.*, who also detected a decrease in both immunoglobulins levels. They stated that in primary immunodeficiency, antibody deficiencies were the most common, constituting 53% of the patients (23).

Flow cytometry is a highly sensitive tool for evaluating the immune system and supporting the diagnosis of Kanegane *et al.* (24). This study showed a statistically significant decrease in CD3%, CD8% and CD19% in group II as compared to group I. Stepenskyet *et al.* found a decrease in CD4% and not CD16% in their study done on severe combined immunodeficiency (23).

There is significant heterogeneity in circulating subsets in blood as per Bisgin *et al.* (25). Immunophenotyping of lymphocyte subsets by flow cytometry is of great utility in the diagnosis and prognosis of many PIDs (24). In addition to immunological phenotype, more specific gene panels may be targeted for diagnosis when immune profiling is not informative enough.

In this study, the most common immunophenotyping patterns found in group II, in thirty cases, were T – B –NK+ and T – B + NK+ patterns. This agrees with Aluri *et al.*, who found the same immune phenotyping patterns in their studied cases (17). Picard *et al.* stated that difficulties are met in these conditions regarding the clinical and immunological presentation. This is due to extensive phenotypic variability for individual gene defects. This suggests that host and/or environmental modifying factors affect phenotype between individuals who share the same mutation within the same gene. Nevertheless, a variety of mutations within the same gene add to disease complexity (26). Genetic, clinical, and immunological heterogeneity add to the complexity of PID diagnosis. However, multiplex gene testing in a single assay helps solve these complexities. This came with the introduction of next-generation sequencing that provided better screening and diagnosis for conditions with genetically heterogeneous backgrounds (8). Babies diagnosed with SCID and treated through transplantation, enzyme replacement, and/or gene therapy survived in 92% of the cases (27). Newborn screening for SCID enables early diagnosis in the asymptomatic phase (28).

This study revealed molecular variants in lymphoid-specific recombinase activating genes *RAG1* and *RAG2* genes in 18 of 30 cases (60%) while De-Pagter *et al.*found molecular variants in *RAG1* and *RAG2* genes in 26% of their studied cases. In the current study, Sanger sequencing was used to detect disease-causing mutations. E880K, A960A, H249R, and K820R were previously reported variants in the *RAG1* gene: they were recognized in a heterozygous state through SCID patients. E880K was reported as a disease-causing variant, while A960A, H249R, and K820R were reported as polymorphisms (29).

S913R and V782G are new variants in the *RAG1* gene that were recognized in heterozygous states in SCID patients; both S913R and V782G are predicted to be disease-causing variants.

P501T and rs10836573 were previously reported variations in the *RAG2* gene which were recognized through SCID patients. P501T was reported as a disease-causing variant, while rs10836573 is a polymorphism (29).

L514M and cDNA.2129A>T are new variants in the *RAG2* gene that were recognized in SCID patients, they are predicted to be polymorphism. (29).

This study revealed 18 cases (from 30 sequenced patients' samples) with identified variants (mutation or polymorphism) in *RAG1* and *RAG2* genes, this agrees with Bisgin *et al.*, who studied 37 suspected PID patients and identified the causative mutations in 17 of them (25). However, some suspected subjects were undiagnosed by immunophenotyping and immunoglobulin profiles. Researchers recommended whole exome sequencing to reveal uncharacterized mutations in other genes and highlight other genetic etiologies. Also, Woon and Ameratunga found that 23% of suspected cases had a positive genetic diagnosis (30).

The study has some limitations. PIDs are rare and suspicions depend on warning signs (12). These signs are not necessarily recognized in infancy but start appearing with age (31). This is the reason why, in the postnatal diagnosis group, many patients experienced serious infections. Due to inadequate prophylaxis and treatment, irreversible complications took place which increased morbidity and mortality.

Positive family history for consanguinity in 57% of cases was recorded in this study. In the immune deficiency disorder research field, populations with high consanguinity create a high-potential area that facilitates the detection of new genetic causes. Causative genes for many types of SCID have been identified along the path of the post-genome era. It is expected that more is yet to come. Using such knowledge and expanding the understanding of disease pathogenesis remains the ultimate objective. An objective that assures research continuity is to improve diagnostic methods and develop an effective treatment for affected individuals (32).

In conclusion, this study demonstrated 18 SCID cases from 30 sequenced samples with identified variants (mutation or polymorphism) in the *RAG1* and *RAG2* genes which revealed the importance of full evaluation of patients with SCID.

The main challenge remains in documenting genetic defects in patients with SCID. This study affirms the importance of the *RAG1* and *RAG2* genes for enhancing the efficiency of the recombination reaction. However, further studies involving other genes that could be the disease-causing of SCID patients are recommended.

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Contributions

NK, HA: conceptualization, methodology. EA, NE: investigation. AA, RG: formal analysis. AF, AR: writing – original draft. SK, IH: writing – review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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A real-life cohort of mepolizumab treatment in severe eosinophilic asthma

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Key words

Asthma; anti-IL5; oral systemic corticosteroids; mepolizumab; eosinophils.

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Impact statement

Our results confirm the efficacy and safety of mepolizumab in routine clinical practice and we observed the significant decrease of ECP, suggesting a reduction in eosinophil activity following mepolizumab treatment.

Summary

Background. *Mepolizumab, a monoclonal antibody that interacts with IL-5, was the first anti-IL-5 approved for uncontrolled severe eosinophilic asthma. In several randomized, placebo-controlled trials, treatment with mepolizumab has shown a significant improvement in asthma symptoms and the need to use of oral corticosteroids (OCS). Several studies have correlated blood levels of eosinophil cationic protein (ECP) with the degree of eosinophilic inflammation, which could make it an indirect marker of eosinophilic activity.* ***Methods.*** *This was a single-center retrospective study that included all patients diagnosed with severe eosinophilic asthma under treatment with mepolizumab. We recorded the number of exacerbations, daily prednisone intake, asthma control test scores and forced expiratory volume in the first second.* ***Results.*** *We followed 22 patients, 14 of whom were OCS-dependent with a mean daily dose of 15.85 ± 15.62 mg prednisone. After 12 months, only five continued taking OCS and the mean daily dose was reduced by up to 2.50 ± 3.84 mg ($p < 0.007$). The exacerbation rate at baseline was 2.91 ± 2.27 and decreased to 0.82 ± 1.14 in the following year ($p < 0.001$). ACT scores increased significantly from 16.00 ± 5.85 to 20.71 ± 4.45 after six months ($p = 0.003$). We also observed a decrease in ECP from 81.46 ± 43.99 µg/L to 19.12 ± 18.80 µg/L ($p > 0.001$).* ***Conclusions.*** *These real-life results are consistent with previous clinical trials demonstrating the efficacy and safety of mepolizumab in routine clinical practice for severe uncontrolled eosinophilic asthma. We observed a significant decrease in blood eosinophil counts and in ECP levels, suggesting a reduction in eosinophil activity following mepolizumab treatment.*

Introduction

Asthma is a common chronic airways disease characterized by variable respiratory symptoms and airflow limitation (1). The worldwide prevalence ranges between 1% and 18%, being highest in developed countries (2). Approximately 4% of this total adult asthma population have a severe asthma phenotype, characterized by difficulty in achieving disease control despite high-dose inhaled corticosteroids combined with long-acting b2-agonists (LABAs) or oral corticosteroids (OCS) (3, 4). Such patients may need another type of treatments, such as the new biologic therapies targeting different pathways.

Since omalizumab, the first commercialized biological therapy targeting Immunoglobulin (Ig)E, several antibodies have been developed in the last few years to treat asthma driven by type-2 inflammatory mechanisms, characterized by the production

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of type-2 cytokines: interleukin (IL)-4, IL-5 and IL-13. IL-5 plays a key role in stimulating the production, recruitment and activation of eosinophil granulocytes (5). Mepolizumab, a humanized IgG1/k monoclonal antibody that interacts with IL-5 by blocking the binding of this cytokine to the alpha subunit of its receptor (6), was the first IL-5 antibody approved by both the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) for uncontrolled severe eosinophilic asthma. In several randomized placebo-controlled trials, treatment with mepolizumab has shown a significant reduction in exacerbation rates, as well as in the intake of oral corticosteroids and an improvement in the quality of life of these patients (7, 8). Until now, a blood eosinophil count of ≥ 150/μL is practically the only available biomarker for selecting asthma patients who would benefit from treatment with mepolizumab (9). In addition, greater treatment effects with increasing blood eosinophil count have been found. Valid biomarkers in asthma are currently needed to provide information about diagnosis, prognosis, clinical outcomes, and other clinical features, but only a few of them have been described and characterized to date (*e.g.*, IgE, fractional exhaled nitric oxide [FeNO], periostin) (10-12). Several studies have correlated blood levels of eosinophil cationic protein (ECP) to the degree of eosinophilic inflammation, which could make it an indirect marker of eosinophilic activity (12, 13) but this needs further validation in larger populations with severe asthma, its prize is similar to Immunoglobulin E test and ECP could be an additional test to include in a laboratory protocol in an asthma clinic.

Few studies have been published to date about real-life experiences and possible criteria for treatment response with mepolizumab. Our aim was to evaluate severe eosinophilic asthma patients treated with mepolizumab in real-world practice and to find potential predictors of response.

Materials and methods

Our single-center retrospective study included all patients diagnosed with severe eosinophilic asthma with inadequate control despite treatment with high-dose inhaled corticosteroids (ICS) combined with long-acting beta 2 agonists (LABA) that had started treatment with mepolizumab, according to its current indication in Spain. They were followed up for at least one year in our difficult-to-control asthma unit at the La Paz University Hospital in Madrid, Spain. For inclusion, patients had to have a blood eosinophil count of at least 150 eosinophils/μL at screening, and/or at least 300 eosinophils/μL at any point in the previous year. Most of our patients had received continuous or near-continuous treatment with OCS.

Patients received subcutaneous injections of mepolizumab 100 mg every four weeks over a period of at least 12 months. Throughout this treatment period, the following parameters were recorded at baseline, 4-6 months and 12 months: number of asthma exacerbations, daily intake (mg) of prednisone, forced expiratory volume in the first second (FEV_1), blood eosinophil count, ECP levels, total serum IgE levels, FeNO and asthma control test (ACT) scores. At each injection appointment, patients were asked about the occurrence of adverse effects and subsequently registered.

Statistical analysis

Statistical analysis regarding categorical variables was stated as absolute numbers (n) and percentages (%), and continuous variables as means ± standard deviation (SD).

Table I *- Baseline demographic and clinical characteristics of severe asthmatics patients.*

Characteristics	Mepolizumab patients (n = 22)
Age (years)	55.77 ± 15.03
Female, n (%)	15 (68)
Smoking history, n (%)	
Active smoker	1 (5)
Former-smoker	10 (45)
Non-smoker	11 (50)
Body mass index	25.39 ± 5.34
Continuous OCS therapy, n (%)	14 (64)
Mean daily dose of prednisone or equivalent at baseline (mg)	15.85 ± 15.62
Blood Eosinophils (cells/ml)	790.91 ± 549.96
FEV_1 (l) (pre-BD)	1.93 ± 0.88
FeNO (ppb)	59.99 ± 63.17
Total IgE (kU/L)	214.96 ± 211.54
ECP (μg/L)	81.47 ± 43.99
ACT	16.00 ± 5.85
Comorbidities, n (%)	
Rhinitis	14 (64)
Rhinosinusitis	17 (77)
Nasal polyps	12 (55)
Gastroesophageal reflux	10 (46)
Bronchiectasis	16 (73)
Obstructive sleep apnea syndrome	7 (32)
Aspirin-exacerbated respiratory disease (confirmed)	2 (9)
Psychiatric disorder (depression/anxiety)	6 (27)
Asthma and COPD overlap (ACO)	1 (5)
Eosinophilic granulomatosis with polyangiitis (confirmed)	1 (5)

ACT: asthma control test; BD: bronchodilator; COPD: chronic obstructive pulmonary disease; ECP: eosinophil cationic protein; FeNO: fractional exhaled nitric oxide; FEV1: forced expiratory volume in the first second; IgE: immunoglobulin E; OCS: oral corticosteroids.

The evolution of parameters of interest was calculated using generalized linear models. A P-value < 0.05 was considered statistically significant. Data analysis was performed with SAS 9.3 (SAS Institute, Cary, NC, USA).

Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The protocol was approved by local regulatory ethics committee (PI-2932). Information revealing the patient's identity has been avoided. All patients have been identified by numbers or aliases and not by their real names.

Ethics approval was not required because it was a retrospective and observational study. We did not change our daily clinical practice.

The study participants have given their written informed consent to publish their case.

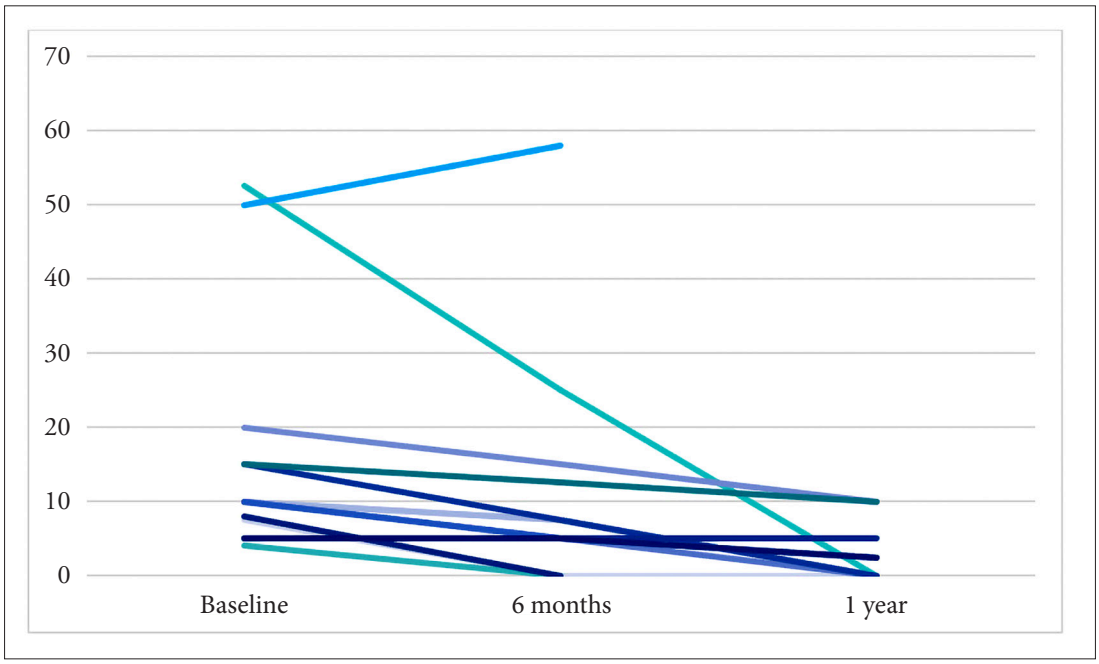
Results

We followed 22 patients with severe eosinophilic asthma in our difficult-to-control asthma unit, starting treatment with mepolizumab. Three patients (13.64%) discontinued treatment before completing 12 months. One of them developed herpes zoster after the second injection of mepolizumab, which resolved properly without sequelae. We believed that this was probably related to the drug. Another patient suffered from non-specific arthralgias that were not considered clearly related to mepolizumab, and the last case involved a non-responder patient after the eighth administration. Other mild side effects, sporadically reported by our patients, included headache, low back pain and local irritation at the injection site.

Demographic and clinical characteristics of all patients, including comorbidities, are shown on **table I**. In this cohort, rhinitis and chronic rhinosinusitis with nasal polyps were the most frequent comorbidities, with a high prevalence of bronchiectasis (73%). Thirteen patients had previously received omalizumab without success.

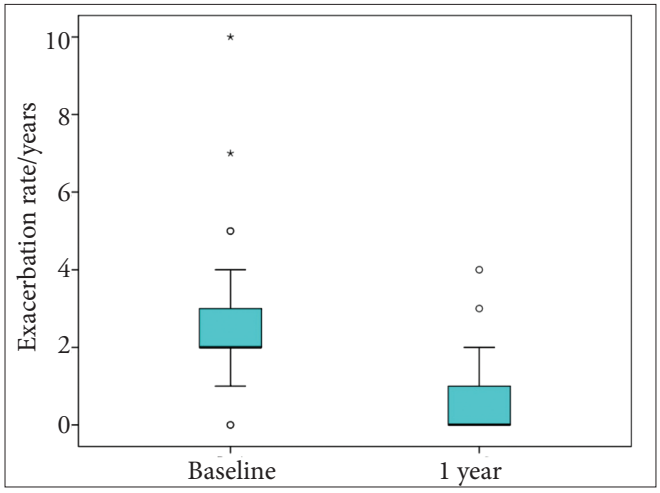
Prior to mepolizumab treatment, 14/22 patients were OCS-dependent, with a mean daily dose of 15.85 ± 15.62 mg prednisone or equivalent. After 12 months, only 5/22 continued taking OCS and the mean daily dose was reduced by up to 2.50 ± 3.84 mg ($p < 0.007$) (**figure 1**). The exacerbation rate at baseline was 2.91 ± 2.27 and decreased to 0.82 ± 1.14 in the following year ($p < 0.001$) (**figure 2**). The variation of different clinical parameters from baseline to month 6 and 12 is shown in **table II**. Disease control measured by ACT scores significantly increased from 16.00 ±5 .85 to 20.71 ± 4.45 after six months ($p = 0.003$), although a statistically significant change was not obtained at the 12-month follow-up visit. Regarding lung function, FEV_1 showed an average increase of

Figure 1 *- Effect of mepolizumab on OCS intake.*



Mean daily prednisone doses (mg) assessed at baseline, six months and after a year of treatment (p < 0.007).

Figure 2 *- Exacerbation rate at baseline and after a year of treatment with mepolizumab (p < 0.001).*



238.85 mL after 12 months of treatment, without reaching statistically significant differences compared to baseline (p = 0.261). FeNO measurements increased from a mean of 59.99 ± 63.17 parts per billion (ppb) to 73.23 ± 48.28 ppb after 12 months (p = 0.604). All these findings are consistent with a dramatic reduction in the blood eosinophil count after one year, from 790.91 ± 549.96 cells/ml to 98.00 ± 90.73 cells/ml (p > 0.001). We also observed a significant decrease in ECP, from 81.46 ± 43.99 µg/L to 19.12 ± 18.80 µg/L, parallel to the reduction in peripheral blood eosinophil count (p > 0.001). We observed no significant differences in total IgE at 6 or 12 months.

Discussion and conclusions

These real-life results are consistent with previous clinical trials showing a clear benefit of this biologic therapy in patients with severe eosinophilic asthma not adequately controlled with high-dose ICS-LABA and/or OCS. A recent article by Bagnasco *et al.* (14) compared the baseline characteristics of patients treated with mepolizumab in real life with those patients who took part in the main regulatory trials, finding several differences such as improved lung function, higher eosinophil count and treatment with lower doses of prednisone in the former group. This real-world experience is important to support the results of clinical trials and to include new knowledge from clinical practice in guidelines.

The efficacy and safety of mepolizumab have been reported in several clinical trials. In the DREAM study (7), an intravenous formulation resulted in a 48% reduction in clinically significant exacerbations in the active arm *versus* placebo. In fact, high blood eosinophil counts have been found to play an important role in the risk of asthma exacerbations (15, 16); therefore, one of the key therapeutic goals for these types of drugs is to maintain blood eosinophil numbers below relevant thresholds. In our patients, we observed a significant decrease in blood eosinophil counts and in ECP levels, suggesting a reduction in eosinophil activity. However, there is not sufficient evidence to suggest that a decrease in ECP levels is related to a better response to mepolizumab (17). Regarding lung function, the DREAM study confirmed some other early studies in which there was no significant change in FEV1 during treatment (7, 18, 19), as was observed in our patients. More recent clinical trials such as MENSA (8) and MUSCA (20) reported increases of 98 and 120 mL in FEV1 when comparing patients treated with mepolizumab with placebo. In terms of the steroid-sparing effect, the SIRIUS study showed a median percentage reduction of 50% in the glucocorticoid dose in the mepolizumab group, compared to no reduction in the placebo group (21). Similarly, in our study, the number of OCS-dependent patients dropped from 14 to five after one year of treatment. Results from the clinical trials are consistent with those in patients treated with mepolizumab in real life. Rhinitis and nasal polyps were the most frequent comorbidities in our study, as was reported by Bagnasco *et al.* (22), although we observed a higher prevalence of bronchiectasis (73%) in our patients, we think it is due to our Asthma Clinic Protocol where every patient is studied with chest computed tomog-

Table II *- Variation of different clinical parameters from baseline to month 6 and 12.*

	Peripheral blood eosinophil count (cells/ml)	ECP (µg/L)	Total IgE (kU/L)	ACT	FEV_1 (ml)	FeNO (ppb)
Baseline	790.91 ± 549.96	81.47 ± 43.99	214.96 ± 211.54	16.00 ± 5.85	1,930.41 ± 880.29	59.99 ± 63.17
6 months	114.00 ± 99.07	22.38 ± 19.08	128.12 ± 128.77	20.71 ± 4.45	2,295.63 ± 634.61	54.95 ± 43.45
12 months	98.00 ± 90.73	19.12 ± 18.80	160.26 ± 108.57	18.50 ± 5.27	2,169.26 ± 677.75	73.23 ± 48.28

All data are expressed in mean and standard deviation (SD); ECP; eosinophil cation protein; ACT: asthma control test; FEV1 (ml): exhaled expiratory volume during the first second; FeNO: fractional exhaled nitric oxide.

raphy, however, not all bronchiectasis presented clinical implications in asthma. In 2020, we published a retrospective study (23) performed in patients of our difficult-to-control asthma unit obtaining a similar prevalence of bronchiectasis (60.4%) measured by chest computed tomography. Regarding other comorbidities, one of our patients had a confirmed diagnosis of eosinophilic granulomatosis with polyangiitis (EGPA) that was satisfactorily controlled with mepolizumab 100 mg every four weeks, despite the results of several open-label or placebo-controlled studies where the effectiveness in the induction and maintenance of remission in EGPA was under mepolizumab 300 mg subcutaneously once a month (23-25).

Some real-world studies evaluating the efficacy of mepolizumab, such as Pelaia *et al*. (27), have demonstrated a significant reduction in the annual asthma exacerbation rate (from 3 to 0), with a concomitant reduction in OCS doses (from 6.25 mg/day of prednisone to 0 mg/day), which is similar to our findings. The REDES study (27), a multicenter trial that evaluated mepolizumab efficacy in eosinophilic asthma, found that exacerbation rates decreased by 77.5% and that 50.6% of patients did not suffer any exacerbations during the 12 months of treatment. In addition, 47.8% of the patients were able to discontinue OCS treatment and the mean daily dose was decreased by 59.9%. In turn, the REALITI A study (28) found a reduction in exacerbation rates of 69%, from 4.63 to 1.43 ($p < 0.001$) and the daily OCS dose decreased from 10 mg/day pre-treatment to 5 mg/day after 21 weeks.

A potential cause of discrepancies with these anti-IL5 drugs could be the assessment of treatment response. Global Initiative for Asthma (GINA) Guidelines recommend re-evaluating treatment response to adjust medications according to response (29). Some authors propose the establishment of response criteria for evaluation of treatment. Drick *et al*. (31) recommended an initial follow-up of six months, in order to identify response criteria. In this study patients were divided into two groups: responder and non-responder, having as a primary criterion the improvement of subjective condition (including their subjective asthma-related symptoms, quality of life, number of exacerbations and improvement of physical fitness), as well as lung function and eosinophils in peripheral blood. Some scientific societies have proposed a response-criteria based on previous studies (31).

In real-world settings, only approximately 30% of severe asthma patients are OCS-dependent, with many patients using OCS in short courses of treatment rather than continuously (32). In our study, only one patient discontinued treatment with mepolizumab due to lack of efficacy, and that was after the eighth dose. In this patient, we observed a notable decrease in the peripheral eosinophil count, therefore this biomarker should not be used in isolation for response assessment. Consistent with previous studies and clinical trials, adverse events were rare and mostly mild in our study, allowing most of the patients to complete treatment for at least a year (33). We measured inflammatory biomarkers such as FeNO and serum ECP, finding different behaviors, but no significant association with clinical improvement was observed. Unlike some previous studies, we did not observe significant improvement in lung function.

Although the small population size is one of the major limitations of this study, our results reinforce those previously reported in regulatory clinical trials and in the few published real-world studies.

In conclusion, the results from our single-center study confirm the efficacy and safety of mepolizumab in routine clinical practice. As reported in previous trials and real-life setting studies, we observed a significant decrease in peripheral blood eosinophilia, asthma exacerbations, OCS intake and an improvement in asthma control, accompanied by the excellent safety profile and tolerability of the drug.

Fundings

None.

Contributions

All authors contributed equally to this work.

Conflict of interests

The authors declare that they have no conflict of interests.

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IgE-mediated reactivity to non-specific lipid transfer protein (nsLTP): clinical implications and management – a consensus document of the Association of Italian Territorial and Hospital Allergists and Immunologists (AAIITO)

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Impact statement

IgE-mediated reactivity to nsLTP is the primary cause of adult-onset food allergy in Mediterranean countries. Effective management requires tailored strategies, including avoidance, testing, and expert guidelines.

Summary

The primary cause of adult-onset food allergy in Mediterranean countries is IgE-mediated reactivity to non-specific Lipid Transfer Protein (nsLTP), with a prevalence of 9.5% in Italy. nsLTP is heat- and pepsin-stable due to its 3D structure, causing severe allergic reactions, even anaphylaxis. It's conserved across plants and a "panallergen" due to homologous forms in various vegetable foods. Found in Rosaceae fruits' skin, it's categorized into nsLTP1 (9 kDa) and nsLTP2 (7 kDa), representing 93% and 7% of the molecules described to date, respectively. Pru p 3 (nsLTP1) from peach is a primary sensitizer, binding more epitopes than other homologs. Cross-reactivity varies in sensitized patients, influenced by IgE levels. Clinical manifestations range from none to various symptoms. Managing patients sensitized to nsLTP without clinical allergy is a challenge. Sensitization hierarchy usually starts with peach, then expands through Prunoideae, Rosaceae, and other foods. Clinical symptoms don't always expand across LTPs. Patients can tolerate some nsLTP-containing foods and consuming them may maintain tolerance. The absence of guidelines led to the Associazione Allergologi Immunologi Italiani Territoriali e Ospedalieri (AAIITO) creating a consensus-based document. Strategies involve avoidance, self-injectable adrenaline, verification through in vivo and in vitro testing, considering cofactors, and peeling fruits. In localized reactions, abstinence is recommended if specific IgE is high. Concurrent pollinosis may complicates diagnosis, but may help management since symptoms are often less severe. Asymptomatic patients are advised to continue normal diets while considering cofactors and total IgE levels. Management strategies should be case-specific, based on expert Consensus Document.

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Consensus document

IgE-mediated reactivity to non-specific Lipid Transfer Protein (nsLTP) stands as the main cause of primary food allergy in adults in Mediterranean countries (1-3), with a prevalence of 9.5% Pru p 3 reactivity observed in Italy (4). The heat- and pepsin-stability due to its three-dimensional structure featuring 4 α-helices linked by 4 disulfide bridges (5) make it an allergen capable of inducing severe allergic reactions, even leading to anaphylaxis (6). nsLTP is a highly conserved protein from a phylogenetic standpoint (7) and is widely distributed throughout the plant kingdom, playing a defensive role against fungi and bacteria (8). As a consequence, homologous forms of nsLTP are present in a broad range of foods, often botanically unrelated to each other, warranting its classification as a "pan allergen" (9-12) (**figure 1**).

This protein is primarily found in the skin of Rosaceae fruits, encompassing numerous fruit-bearing trees (apple, pear, cherry, peach, plum, almond, medlar, rowan, apricot, quince, and more-13). Based on the molecular weight, two subfamilies have been identified: nsLTP1 (9 kDa) and nsLTP2 (7 kDa-8) (14), though 51 out of the 55 molecules officially registered in the WHO/IUIS database (www.allergen.org) belong to nsLTP1 (**table I**). Examples of nsLTP1s include Pru p 3 from peach (15), Mal d 3 from apple (16), Cor a 8 from hazelnut (17), Ara h 9 from peanut (18), Jug r 3 from walnut (19), and Tri a 14 from wheat (20) (**figure 1**). Pru p 3 is regarded as the most likely primary sensitizer to the nsLTP family showing the largest repertoire of IgE-binding epitopes (21), in contrast to other homologous proteins found in different plant sources (22, 23). This view is supported by *in vitro* experiments showing that the pre-absorption of sera of LTP hypersensitive patients with peach LTP almost invariably completely inhibits IgE reactivity to other plant food sources (22). Pollen allergens such as Par j 1 and Par j 2 from *Parietaria* and Ole e 7 from olive tree do not cross-react with either nsLTP1 from plant foods or with each other (24), although they frequently score positive in peach-allergic patients (25-27). LTP2 proteins are cross-reactive with each other but have low sequence identity when compared to LTP1. The widespread presence of nsLTP in nature leads to a long list of foods linked to anaphylactic reactions (28).

For sensitized patients, the risk of cross-reactivity varies significantly from case to case, primarily depending on specific IgE levels for Pru p 3 (22, 29, 30). Another unique characteristic of nsLTP sensitization is the variable spectrum of clinical manifestations resulting from the ingestion of foods containing this protein. Symptoms can range from complete absence to oral allergy syndrome, contact urticaria, asthma, hives/angioedema, and food-dependent exercise-induced anaphylaxis (31).

Figure 1 *- Taxonomic relationships among the non-specific Lipid Transfer Proteins (nsLTPs) currently documented in the literature, which are also catalogued in the International Union of Immunological Societies (IUIS) Database.*

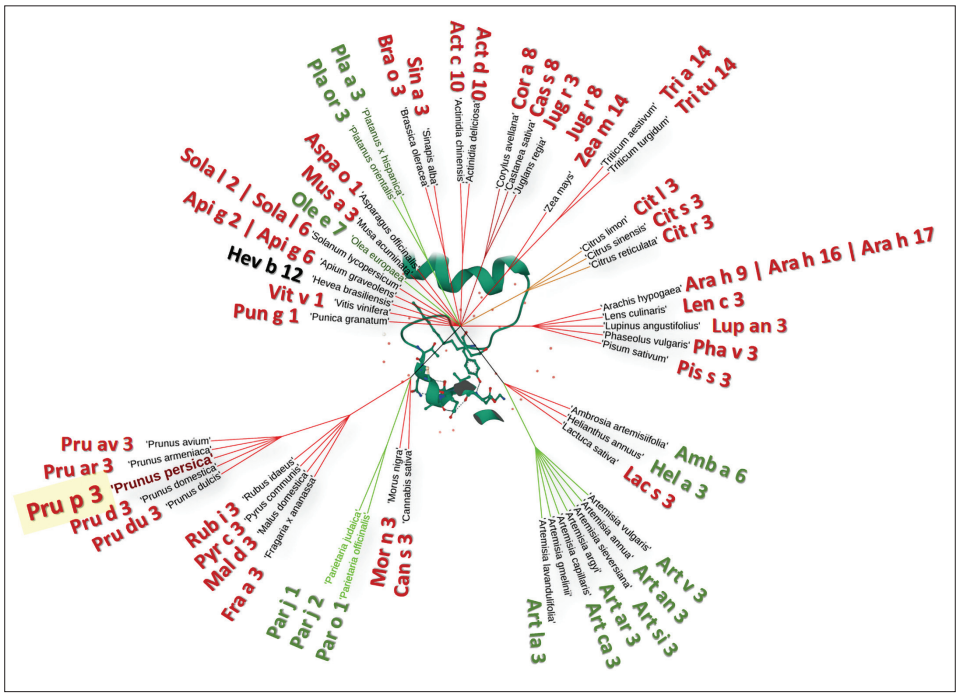


Table I *- List of nsLTPs currently reported by the WHO/IUIS Allergen Nomenclature Sub-Committee.*

	Allergen	nsLipid transfer protein	PM (kDa)	Latin name	Common name
1	Act c 10	nsLTP1	10 kDa	*Actinidia chinensis*	Gold kiwi fruit
2	Act d 10	nsLTP1	10 kDa	*Actinidia deliciosa*	Green kiwi fruit
3	Amb a 6	nsLTP1	10 kDa	*Ambrosia artemisiifolia*	Short ragweed
4	Api g 2	nsLTP1	9 kDa	*Apium graveolens*	Celery
5	Api g 6	**nsLTP2**	7 kDa		
6	Ara h 9	nsLTP1	9.8 kDa		
7	Ara h 16	**nsLTP2**	8.5 kDa	*Arachis hypogaea*	Peanut, groundnut
8	Ara h 17	nsLTP1	11 kDa		
9	Art an 3	nsLTP1	10 kDa	*Artemisia annua*	Sweet Wormwood
10	Art ar 3	nsLTP1	10 kDa	*Artemisia argyi*	Silvery wormwood
11	Art ca 3	nsLTP1	10 kDa	*Artemisia capillaris*	Wormwood
12	Art gm 3	nsLTP1	10 kDa	*Artemisia gmelinii*	Russian wormwood
13	Art la 3	nsLTP1	10 kDa	*Artemisia lavandulifolia*	Mugwort
14	Art si 3	nsLTP1	10 kDa	*Artemisia sieversiana*	Sieversian wormwood
15	Art v 3	nsLTP1	12 kDa	*Artemisia vulgaris*	Mugwort, wormwood
16	Aspa o 1	nsLTP1	9 kDa	*Asparagus officinalis*	Asparagus
17	Bra o 3	nsLTP1	9 kDa	*Brassica oleracea*	Cabbage and others
18	Can s 3	nsLTP1	9 kDa	*Cannabis sativa*	Indian hemp
19	Cas s 8	nsLTP1	9 kDa	*Castanea sativa*	Chestnut
20	Cit l 3	nsLTP1	9.6 kDa	*Citrus limon*	Lemon
21	Cit r 3	nsLTP1	9 kDa	*Citrus reticulata*	Tangerine
22	Cit s 3	nsLTP1	9.46 kDa	*Citrus sinensis*	Sweet orange
23	Cor a 8	nsLTP1	9 kDa	*Corylus avellana*	Hazelnut
24	Fra a 3	nsLTP1	9 kDa	*Fragaria ananassa*	Strawberry
25	Hel a 3	nsLTP1	9 kDa	*Helianthus annuus*	Sunflower
26	Hev b 12	nsLTP1	9 kDa	*Hevea brasiliensis*	Para rubber tree | latex
27	Jug r 3	nsLTP1	9 kDa	*Juglans regia*	English walnut
28	Jug r 8	**nsLTP2**	9 kDa		
29	Lac s 1	nsLTP1	9 kDa	*Lactuca sativa*	Cultivated lettuce
30	Len c 3	nsLTP1	9 kDa	*Lens culinaris*	Lentil
31	Lup an 3	nsLTP1	11 kDa	*Lupinus angustifolius*	Narrowleaved blue lupin
32	Mal d 3	nsLTP1	9 kDa	*Malus domestica*	Apple
33	Mor n 3	nsLTP1	10 kDa	*Morus nigra*	Mulberry
34	Mus a 3	nsLTP1	9 kDa	*Musa acuminata*	Banana
35	Ole e 7	**putative** nsLTP1	9.5 kDa	*Olea europaea*	Olive Tree
36	Pha v 3	nsLTP1	8.8-9.0 kDa	*Phaseolus vulgaris*	Green bean, French bean
37	Pis s 3	nsLTP1	9.5 kDa	*Pisum sativum*	Pea
38	Pla a 3	nsLTP1	10 kDa	*Platanus acerifolia*	London plane tree
39	Pla or 3	nsLTP1	11 kDa	*Platanus orientalis*	Oriental plane tree



	Allergen	nsLipid transfer protein	PM (kDa)	Latin name	Common name
40	Pru ar 3	nsLTP1	9 kDa	*Prunus armeniaca*	Apricot
41	Pru av 3	nsLTP1	10 kDa	*Prunus avium*	Sweet cherry
42	Pru d 3	nsLTP1	9 kDa	*Prunus domestica*	European plum
43	Pru du 3	nsLTP1	9 kDa	*Prunus dulcis*	Almond
44	Pru p 3	nsLTP1	10 kDa	*Prunus persica*	Peach
45	Pun g 1	nsLTP1	9 kDa	*Punica granatum*	Pomegranate
46	Pyr c 3	nsLTP1	9 kDa	*Pyrus communis*	Pear
47	Rub i 3	nsLTP1	11 kDa	*Rubus idaeus*	Red raspberry
48	Sin a 3	nsLTP1	12.3 kDa	*Sinapis alba*	Yellow mustard
49	Sola l 3	nsLTP1	9 kDa		
50	Sola l 6	**nsLTP2**	7 kDa	*Solanum lycopersicum*	*Tomato*
51	Sola l 7	nsLTP1	12.5 kDa		
52	Tri a 14	nsLTP1	9 kDa	*Triticum aestivum*	Wheat
53	Tri tu 14	nsLTP1	9.2 kDa	*Triticum turgidum*	Durum wheat
54	Vit v 1	nsLTP1	9 kDa	*Vitis vinifera*	Grape
55	Zea m 14	nsLTP1	9 kDa	*Zea mays*	Maize

In daily clinical practice, problems arise where a patient sensitized to nsLTP presents without a history of adverse reactions following the consumption of plant-based foods. Similarly, a patient reporting allergic reactions triggered by nsLTP (often to peach, a key allergenic food for this protein) (22) may show cross-sensitization to a wide array of plant-based foods that are tolerated and commonly consumed. It has been demonstrated that some patients allergic to peach but positively reacting in skin tests and specific serum IgE to other Prunoideae tolerate the ingestion of these fruits, suggesting sensitization without clinical allergy (32). Similar findings have been observed in a pediatric population where sensitization to specific plant nsLTPs did not necessarily correlate with symptoms upon food ingestion (33).

Furthermore, the clinical expression of nsLTP sensitization may sometimes require cofactors. Concurrent use of non-steroidal anti-inflammatory drugs (NSAIDs) alongside nsLTP-containing foods can exacerbate adverse reactions (25, 34). nsLTP is the primary cause of exercise-induced anaphylaxis triggered by food in Italy (31). Reactivity may also depend on the simultaneous presence or absence of different foods in the digestive tract (35). Conversely, co-sensitization to PR10 and Profilin, or both, appears to play a protective role, resulting in a lower frequency of severe systemic reactions (25, 36), despite an increased prevalence of first-degree oral allergy syndrome (27). Another protective factor is the presence of atopic dermatitis, where despite very high IgE levels and multiple LTP recognition, the frequency of severe reactions is significantly lower than observed in allergic patients without atopic eczema (25).

Sensitization to LTP appears to follow precise hierarchical pathways, starting from peach, progressing through other Prunoideae (apricot, plum, cherry), Rosaceae (apple, pear), then moving on to walnut, hazelnut, peanut, and subsequently rice, maize, and a wide variety of botanically unrelated plant foods (37). There is also a correlation between specific IgE levels and the likelihood of systemic allergic reactions (36-38). It should also be emphasized that such hierarchy is not rigid but may vary depending on the cases and patients (37).

It has been clinically observed that patients do not tend to progressively expand their clinical symptoms across different LTPs, but rather exhibit the development of clinical allergy either to Prunoideae and/or tree nuts, or to certain vegetables, or cereals that were formerly tolerated albeit scoring positive on SPT or specific IgE measurement (39). Managing sensitized patients who are clinically non-allergic or only exhibit minimal allergic symptoms represents a significant medico-legal and ethical challenge. Recommending a patient to continue consuming well-tolerated foods or advising them to abstain from foods that tested positive *in vivo* and/or *in vitro* investigations due to the risk of unpredictable allergic reactions upon accidental ingestion, requires careful consideration. It is worth noting that, akin to strategies employed in other types of food allergies where oral tolerance induction is attempted through repeated oral administration, consuming tolerated foods by nsLTP-sensitized individuals might

contribute to maintaining a natural state of tolerance. Conversely, eliminating nsLTP-containing foods could potentially disrupt the tolerance state, leading to systemic reactions after ingestion of previously tolerated but now removed foods (39). The Associazione Allergologi Immunologi Italiani Territoriali e Ospedalieri (AAIITO) recognized the need to address the absence of international and national guidelines on this subject and created this document which seeks to provide clinical guidance based on expert consensus.

Case-by-case Indications for LTP sensitization management

Patient with a history of systemic reaction to one or more plant foods

- Complete avoidance of the responsible food, even if subjected to food industry processing (*e.g.*, commercial fruit juices, jams, desserts), due to nsLTP's resistance to such treatments. Prescription of self-injectable adrenaline only in case of anaphylactic events, especially considering the potential for accidental ingestion (hidden food) and risk factors, following the current Italian legislation.
- In skin prick testing, you can use commercial extracts or fresh foods. When using fresh foods, it's important to pay specific attention to the testing site since Lipid Transfer Proteins (LTPs) are mainly found under the skin's surface, especially under the peel. Peach, apple, peanut, walnut, hazelnut, wheat, tomato, and almond must be tested in all cases. Other plant foods (corn, celery, kiwi, rice, barley, fennel, cabbage, lettuce, legumes, citrus, mustard, pomegranate, *etc*.) can be tested based on clinical history.
- Positively reacting foods that were previously regularly consumed and tolerated can be ingested with the recommendation to avoid them in combination with known cofactors (physical exertion, NSAIDs, alcoholic beverages), to avoid consuming more of them within the same meal or day, and to peel them whenever possible.
- Positively reacting foods that previously induced mild local symptoms (oral allergy syndrome) are preferable to be avoided, especially in the presence of high specific IgE levels and in the absence of a positive reaction to PR-10 and/or profilin. Foods testing negative in both *in vivo* and *in vitro* investigations can be consumed, given the low likelihood of an allergic reaction.
- Given the predominant localization of the protein in the superficial layers of foods, peeling Rosaceae fruits is a preventive measure that allows the introduction of minimal allergenic amounts to maintain gastrointestinal tolerance. Peeling should be done with a very sharp knife to prevent carrying nsLTP into the fruit pulp. Additionally, it's important not to use the same knife for peeling the fruit and cutting the slices to eat, as this can risk transferring LTPs into the pulp. Note that certain fruits (*e.g.*, tomato (40), kiwi, and orange) contain LTP not only in the skin but also in pulp and seeds.

Patient with a history of localized reaction (oral allergy syndrome) to one or more plant foods

- In the presence of high specific IgE levels and in the absence of a positive reaction to PR-10 and/or profilin, abstinence from consuming the food in question is recommended due to the risk of systemic reactions. If the food has been previously tolerated, it can be continued to be consumed, paying particular attention to the cofactors already described.
- In cases of low specific IgE levels, consuming that specific food can continue, with the recommendation to peel it, to avoid known cofactors (physical exertion, NSAIDs, alcoholic beverages), and to avoid consuming it in combination with other LTP-containing foods within the same meal or day.

Patient with localized reaction (oral allergy syndrome) to one or more plant foods with concurrent pollinosis

- Patients with peach LTP allergy, when sensitized to PR-10 and/or profilin, show protection from severe symptoms induced by plant foods (36). This suggests that measuring these two proteins could be beneficial (27). Similar results have been observed in patients allergic to any type of nsLTP, whether from food or pollen (27), and in patients sensitized to LTP in different geographic areas of Italy. In the North-East, co-sensitization to profilins and PR-10 is more common, while in the South, higher Pup 3 levels and monosensitization to LTP are prevalent (41).
- The role of airborne sensitization to LTP is debated. While this route of sensitization is established for peach fuzz (42) or peach field workers (43), the significance of two other pollen nsLTPs, Pla a 3 from plane tree and Art v 3 from mugwort, is less certain. IgE reactivity to these proteins, which present cross-reactive epitopes with Pru p 3, might account for weak Pru p 3 reactivity (especially *in vitro*) without necessarily causing clinical symptoms upon LTP ingestion. Nevertheless, recent studies have observed that sensitization to Ole e 7(26) or Pla a 3 (44) is significantly associated with potential systemic food reactions, suggesting their utility as markers of LTP-related reactivity (45). Furthermore, there has been a suggestion that marijuana may play a role in potential contact or airborne sensitization.
- The presence of concurrent pollinosis can thus easily lead to incorrect conclusions in cases of positive reactions to food extracts containing nsLTP. In such cases, to establish an accurate allergological profile, simultaneous determination of specific IgE for nsLTP (Pru p 3), PR-10 (Bet v 1), profilin (Bet v 2 or Phl p 12), and CCD (MUXF3) is suggested if positivity is found via *in vitro* testing.

Occasional identification of LTP sensitization in entirely asymptomatic patients

Patients are advised to maintain their current lifestyle and continue consuming tolerated foods. Particularly for patients with high specific IgE levels, it might be prudent to avoid cofactors such as physical exertion, NSAID intake, and alcohol consumption. It should also be emphasized that elevated levels of total IgE may lead to higher levels of specific IgE antibodies against a particular allergen, without necessarily being linked to an increased risk of adverse reactions (46).

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Contributions

RA: conceptualization. All authors: writing - original draft, writing - review & editing.

Conflict of interests

ES has received consultant arrangements and speakers' bureau participation from Stallergenes, Thermo Fisher Scientific, and non-financial support from Microarray Diagnostics, Vienna, all outside the submitted work. LC has received honoraria from Malesci, Menarini, Mylan and Thermo Fisher Scientific. DV and RA received honoraria from Thermo Fisher Scientific.

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CASE REPORT | Eur Ann Allergy Clin Immunol | Vol 56, N.4, 183-187, 2024

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Ultrafast regimen for Pru p3 sublingual immunotherapy (SLIT-Peach®) in patients with anaphylactic LTP-Syndrome

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Key words

Ultrafast regimen; lipid transfer proteins; food allergy; rPru p3; sublingual immunotherapy.

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Impact statement

Cluster therapies in sublingual immunotherapy are nowadays our aim to achieve in a short period time the maintenance dose. Mild adverse reactions are observed as oral pruritus. We present this efficient new regimen to induce tolerance to SLIT-peach in one day.

Summary

Sublingual immunotherapy with Pru p3 extract (SLIT-peach®) is used in allergy patients to multiple plant foods to induce tolerance to nonspecific lipid transfer proteins (nsLTP). The aim of this paper is to communicate the efficacy of a new ultrafast regimen. Until now on the initiation regimen lasts four days. We present a number of 22 patients with LTP-syndrome due to ingestion of different vegetable foods sensitized to Pru p3. According to European Academy of Allergy position paper (1) food immunotherapy is indicated when avoidance measures are ineffective, undesirable, or cause serious limitations on patients quality of life. Our patients had an impact on their quality of life (score > 130) before SLIT measured with (2) EuroPrevall Food Allergy Quality of Life Questionnaire (FAQLQ). The ultrafast regimen in one day is achieved in the 95% of our patients. Mild adverse reactions were observed, such as oral pruritus presence in almost all patients. Only one patient (5%) achieved the maintenance dose in two days due to intense oral pruritus. No patients presented systemic reactions. The maintenance dose achieved consists of 4 drops (0.16 ml) from vial number 4 daily. The concentration of Pru p3 in vial number 4 is 50 µg/ml. Four drops a day equals 8 micrograms of Pru p3. This new ultrafast regimen in one day is secure in patients with LTP-Syndrome to induce tolerance to SLIT-peach® (Pru p3 extract).

Introduction

Allergy immunotherapy (AIT) has been practiced for more than a hundred years. Repeated low doses of allergen induce clinical tolerance and improve quality of life in patients with respiratory allergy (3). AIT may increase the remission rate of asthma and sensitization to new allergens (4). The route chosen for immunotherapy with aeroallergens can be oral, subcutaneous or sublingual. Subcutaneous immunotherapy is worldwide extended with respiratory patients in the allergy office. Oral tablets are used in patients with house dust-mite allergy with good results (5). Food allergy immunotherapy has been practiced in the last 10 to 15 years using oral and sublingual route. The prevalence and incidence of food allergy is significantly increasing in recent years. Patients are allergic to different food depending on the area where they live (6). Lipid transfer proteins (LTPs) are a very common panallergen in the Mediterranean area. LTPs are very ubiquitous in the plant kingdom. The resistance to pepsin and to chemical digestion is a quality to produce anaphylactic reactions. Patients with LTP-syndrome are often seen in our of-

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Table I *- SLIT in 4-days (conventional regime) (13).*

Day	Vial	Concentration µg/ml of Pru p3	Drops	Dosage of Pru p3 (µg)	TIME between administration (min)
1	1	0.05	1	0.002	15
			10	0.02	-
	2	0.5	1	0.02	15
			10	0.2	-
2	3	5	1	0.2	15
			10	2	-
3	4	50	1	2	15
			2	4	-
			5	10	-
			10	20	-
4	4	50	20	40	Unique adminitration

Once the dose is achieved, maintenance dose will be administered at home: 5 drops from bottle 4, daily (10 µg of Pru p3). Cumulative dose in the initial phase is: 78,442 µg. The cumulative dose in six months is 1,800 µg of Pru p3.

fice (7). Allergens presented in peach, Prunus persica, are well known. Pru p3 is a nonspecific lipid transfer protein (nsLTP) present in peach and associated to severe systemic symptoms. Patients with LTP-syndrome have IgE against Pru p3 and often experience cross-reactivity to other nsLTP present in other fruits, or nuts, cereals, veggies and legumes. Our patients presented cross-reactivity to walnuts, peanuts, hazelnuts, Rosaceae fruits, lettuce and tomato. Sublingual immunotherapy with SLIT-peach® is a commercial extract of concentrated Pru p3. Patients with LTP-syndrome have often anaphylactic reactions to hidden food allergens (8). Wide dietary restrictions in these patients reduce their quality of life. In this context it is necessary

Table II *- SLIT in 2 days (fast regimen, ref. 12).*

Days	Vial µg/ml	Drops	µg of Pru p3	Time between administrations (min)
1	2	1	0.02	30
	0.5	5	0.1	-
	3	1	0.2	30
	5	3	0.6	-
		5	1	-
	4	1	2	30
		-	-	-
		2	4	-
		3	6	-
		4	8	-
	50	5	10	-
2	4	3	6	60
	50	-	-	-
		5	10	-

Once the dose is achieved maintenance dose will be administered at home: 5 drops from bottle 4, daily (10 µg of Pru p3). Cumulative dose in one day 31.92 µg of Pru p3. The cumulative dose in six months is 1,800 µg of Pru p3.

Table III *- SLIT in one day (ultrafast regimen).*

Vial	Concentration of Pru p3 (µg/ml)	Drops	Total Pru p3 µg	Time between administration (min)
1	0.05	1	0.002	15 min
		10	0.02	
2	0.5	1	0.02	15 min
		10	0.2	
3	5	1	0.2	15 min
		10	2	
4	50	1	2	15 min
		2	4	
		4	8	

Once the dose is achieved, maintenance dose will be administered at home 4 drops from bottle 4, daily. This dose equals 8 µg per day of Pru p3. Cumulative dose in one day: 16.46 µg of Pru p3. The cumulative dose in six months is 1,440 µg of Pru p3.

to use an etiological treatment (immunotherapy) to increase tolerance to ingesting foods involved. Immunotherapy in food allergy is recently approved for peanut in the U.S (9). Several doctoral dissertations have published the improvement of quality of life after sublingual immunotherapy with SLIT-peach® Pru p3. The manufacture's standard initiation protocol for SLIT-peach® has a duration of 4 days (**table I**). A fast regimen has been published with a duration of two days (**table II**) by the group of Pereira in 2019. We propose an ultra-fast regimen in one day (**table III**).

Case presentation

We selected 22 patients with LTP-Syndrome, 15 women and 7 men. Ages are between 19 and 43 years old. All our patients had severe anaphylactic reactions to different plant-foods. Inclusion criteria were unequivocal clinical history of allergy to peach and/or other fruits containing LTP, one or more episodes of anaphylaxis following the ingestion of fruits, nuts or vegetables containing LTP. Oral provocation tests were not performed since all patients had anaphylactic episodes after the ingestion of food containing LTP in the previous year and declined a challenge that could induce a new anaphylaxis. We measured the severity reactions with the ordinal food allergy severity score (oFASS-5). Five of them presented reactions oFASS grade 5 (22%). Fourteen of them presented oFASS grade 4 (63%). The severity score oFASS grade 3 was for 3 patients (13%). Quality of life was measured with the EuroPrevall Food Allergy-Quality of Life Questionnaire (FAQLQ). This consists of a 29 items questionnaire with a maximum of 174 score. The score for quality of life in our patients was between 170 and 131 with a media of 150.5 score. All our patients had a restricted diet due to cross-reactivity. Skin pricks tests and specific IgE against Pru p3 were performed according to the standardized European protocols (10) and results were positive in all patients (diameter papule > 3 mm compared to the negative control). Serum specific IgE to Pru p3 was higher than 3.47 KU/L in all our patients. IgG4 against Pru p3 was measured before inducing tolerance. Monitoring IgG4 is relevant in these patients, especially as it is a biomarker of compliance to treatment in SLIT. Molecular diagnosis under Microarray with Immuno Solid-Phase Allergen Chip (ISAC®) and total IgE, were performed in blood tests in all patients. The results are showed in **table IV**.

Specific serum IgE was positive to seven nsLTPs in 40% of our patients (9 out of 22): Ara h9, Cor a8, Jur r3, Pru p3, Art v3, Ole e7, Pla a3. Seven from 22 patients, 31.8%, were positive to six nsLTP: Ara h9, Cor a8, Jur r3, Pru p3, Art v3, Ole e7. Five of 22 patients, 22.7%, were positive to five ns LTP: Ara h9, Cor a8, Jur r3, Pru p3, Art v3. And only one patient out of 22, 4.5%, was positive to 4 nsLTP: Ara h9, Cor a8, Jur r3, Pru p3. Results are showed in **table IV**. Blood tests were performed before SLIT, with a total IgE between 375 KU/L and 123 KU/L with an average of 249 KU/L and serum IgE against Pru p3 was between 53.9 KU/L and 3.47 KU/L with an average of 28.6 KU/L (**table IV**). All patients underwent the administration in the clinical office of the ultrafast regimen designed in our service according to **table III**.

Results

Twenty-one patients achieved tolerance in one day with our proposed regimen (95.45 %) and only 1 patient needed the rapid regimen of 2 days, for presenting intense oral syndrome, not presenting systemic reactions. None of our patients presented systemic reactions. The total dose of Pru p3 received in one day with the ultrafast regimen is 16.442 µg. The maintenance dose is 8 µg of Pru p3 daily (4 drops).

Table IV *- Clinical data.*

Age	Gender	FASS5	FAQLQ	IgE total	IgE to Pru P3	IgG4 to Pru P3	ISAC (Nº LTP)	Ultrafast	Swallowed
19	Male	Grade 4	137	234	7.23	0.1	7	Yes	Yes
24	Male	Grade 4	131	257	5.65	0.02	6	Yes	Yes
27	Female	Grade 3	146	123	11.34	0.7	6	Yes	Yes
33	Female	Grade 4	165	254	13.4	1.01	7	Yes	Yes
41	Female	Grade 5	170	176	52.08	1.2	7	Yes	Yes
19	Female	Grade 4	166	201	21.5	0.23	7	Yes	Yes
32	Female	Grade 3	142	126	3.47	0.01	6	Yes	Yes
35	Male	Grade 4	153	327	17.23	0.8	7	Yes	Yes
23	Female	Grade 4	146	251	7.34	0.1	7	Yes	Yes
26	Female	Grade 3	161	168	6.74	0.2	7	Yes	Yes
33	Male	Grade 4	142	248	32.7	0.6	6	Yes	Yes
41	Male	Grade 4	139	351	18.7	1.08	7	Yes	Yes
18	Female	Grade 5	146	198	45.8	0.23	5	Yes	Yes
23	Female	Grade 4	153	289	35.8	0.1	5	Yes	Yes
43	Female	Grade 4	153	341	23.9	0.2	5	Yes	Yes
42	Male	Grade 4	162	246	53.9	0.7	6	Yes	Yes
32	Female	Grade 4	137	256	24.1	1.01	4	Yes	Yes
41	Female	Grade 4	131	234	12.8	0.3	5	Yes	Yes
37	Male	Grade 5	166	197	58.9	0.1	6	Yes	Yes
28	Female	Grade 5	170	329	28.91	0.7	7	No/two days	Yes
39	Female	Grade 4	140	247	8.91	0.6	6	Yes	Yes
19	Female	Grade 3	132	375.6	13.4	0.28	5	Yes	Yes

Ordinal food allergy severty score: oFASS-5, grade 1 includes reactions restricted to the oral cavity; grades 2 to 5 may include oral symptoms, but other target organs are affected. Grading is based on the organ/system involved regardless of the type or number of specific symptoms present of that organ/system. Grades 2 and 3 include skin, eye/nose, digestive, and/or uterine involvement, either 1 or more than 1 of them, respectively. Lx and/or BR involvement (even isolated) classifies a reaction as Grade 4, and CV and/or NS involvement (even isolated) as grade 5. In grades 4 and 5, other target organ/systems of lower grades may be affected. oFASS-3 is a simplified version, where mild corresponds to grade 1, moderate to grades 2 and 3, and severe to grades 4 and 5 of the oFASS-5 (14). FAQLQ: Food Allergy Quality of Life Questionaire (total items 29, maximum range 174); ISAC: immuno solid-phase allergen chip. Levels of IgE are measured in KU/L; levels of IgG4 are measured in mgA/L; Serum IgE against 112 allergens. Results are positive for 7 nsLTP: Ara h9, Cor a8, Jur r3, Pru p3, Art v3, Ole e7, Pla a3. None of our patients is sensitized to specific species.

Discussion

Cluster doses in one day are safe and fast to induce tolerance to SLIT-peach® in patients with a history of anaphylaxis to Pru p3. The ultra-fast regimen in one day has already been published (11) as a case report. Our series is a prospective study of 22 patients, with a cumulative dose of 16,442 µg (2 hours and 15 minutes duration) and a daily maintenance dose of 8 mg of Pru p3. The daily application at home proposed makes a cumulative dose of 1,440 µg of Pru p3 in six months. There is another paper with this ultra-fast regimen of administration for peach-ALK slit vaccine that achieves the maintenance dose in one day, but also repeats this dose in a second visit. It has also been published by Pereira *et al.* (12) in 2019. The number of patients in the active group in this case is n = 10. The cumulative dose achieved in one day in this series was 47 µg of Pru p3, this is superior compared to our group. We proposed a modified regimen only in one day and a daily maintenance dose. The aim of this regimen is to reduce the time of hospital administration to 1 day. Monitoring IgG4 is relevant in these patients, it could be a biomarker of compliance to treatment in SLIT.

Conclusions

SLIT with Pru p3 extract is secure in patients with LTP-syndrome even for patients with severe symptoms, such as anaphy-

laxis. Cluster administration in one day is well tolerated. Severity score oFASS does not predict tolerance to SLIT-peach in patients with LTP-syndrome.

Fundings

None.

Contributions

ACM: conceptualization; MSZP: writing - original draft, writing - review & editing. YPP: writing - original draft. JCZ: data curation.

Conflict of interests

The authors declare that they have no conflict of interests.

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Clinical characterization of peach allergic patients and respective molecular sensitization profile with ALEX®2 macroarray

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To the Editor,

lipid transfer proteins (LTP) are plant kingdom panallergens, responsible for a significant number of food allergic reactions, particularly in the Mediterranean area. Pru p3 (peach LTP) is the prototype protein of this family. Symptoms may range from oral allergy syndrome (OAS) to systemic and potentially fatal reactions, like anaphylaxis. Previous studies have found an association between peach allergy and sensitization to pollens from several taxonomically unrelated species, such as *Artemisia*, *Parietaria* or plane tree and probably, in less extent, with olive tree, which are quite common in the Mediterranean area. So, it is appropriate to consider the possibility of a link between inhalation sensitization to LTP pollen and LTP food allergy, namely as a primary route of sensitization to LTP (1-8).
Other panallergens have also been well identified for the study of peach allergy: Pru p1, a pathogenesis-related protein group 10 (PR-10) (homologous to Bet v1); Pru p4, a profilin; and Pru p2, a thaumatin-like protein (4). More recently, two new proteins considered allergenic were identified: Pru p7, a gibberellin-regulated protein (GRP) and Pru p9, belonging to the PR-1 group (5, 6, 9).
The allergens considered major are Pru p3 and Pru p7, both resistant to heat and proteolytic digestion, whose typical allergic symptom is anaphylaxis. Similarly to Pru p3, sensitization to Pru p7 is considered a risk factor for severe allergic reactions to fresh fruit and it seems to be particularly important in patients who are peach-allergic but not sensitized to Pru p1, Pru p3 or Pru p4, which is especially common in areas with high cypress pollen exposure (9-12).
Skin prick tests (SPT) are used in these patient's investigation and follow-up, being validated for reactions' responsible allergens identification and for tolerance acquisition assessment, although sensitivity could vary depending on allergen

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representation (9). *In vitro* study, using molecular components to characterize the sensitization pattern of patients with different phenotypes of the disease is considered relevant in the investigation (12). The identification of sensitization patterns that are associated with the severity of the disease would allow a better follow-up of these patients, limiting excessive dietary restrictions and an adequate selection for specific immunotherapy with Pru p3 (12).

The new Macroarray Allergy Explorer® (ALEX®2) technique (Macroarray Diagnostic, Vienna, Austria) is the first ELISA based *in vitro* multiplex allergy test allowing simultaneous measurement of total IgE (tIgE) and specific IgE (sIgE) for more than 150 total extracts (sIgEte) and more than 100 molecular components (sIgEmc) simultaneously, granting a better interpretation and clinical correlation of the results (13, 14). The use of a carbohydrate determinant inhibitor (CCD) reduces the interpretative burden of CCD positive patients (about 25% of patients have anti-CCD IgE), which are known to be clinically insignificant (15).

In this preliminary study, of a small cohort, we aimed to characterize the clinical and molecular sensitization profile of a population of peach allergic patients with the new ALEX®2 technique and identify possible sensitization profiles associated with severe reactions.

We show a single-center cross-sectional study, involving adult patients with a history of peach allergic reaction, positive SPT and sIgE for peach, who were not undergoing immunotherapy with Pru p3. Patients were subdivided into 2 groups: systemic reaction (SRG) − 13 patients, and local reaction (LRG) − 7 patients. This study protocol was approved by Ethical Committee of Unidade Local Santa Maria and Centro Académico de Medicina de Lisboa, approval number 128/20, and every patient signed a written informed consent. Demographic and clinical data were collected through written survey and SPT with fresh fruits (peach, apple, kiwi, grape, plum, pear and tomato), vegetables (celery), nuts (hazelnut), legumes (peanut), cereals (wheat), pollens (*Artemisia* and *Parietaria*), and macroarray ALEX®2 were performed. SPT were carried out according to food symptoms and related foods, including as many food groups containing LTP as possible.

For statistical analyses (IBM-SPSS software, v25.0), t-independent and Mann-Whitney tests were used to compare parametric and non-parametric independent samples, respectively. Fisher's exact test or Qui-square test were used to evaluate associations between categorical variables, and Cohen's K-test was used to assess agreement. P-values < 0.05 were considered statistically significant.

A total of 20 patients were enrolled in the study, mean age of 28.8 ± 11.1 years old (mean age on the first reaction to peach 18.2 ± 10.3 years old), 16 (80%) were female (**table I**). Almost all patients (n = 14; 70%) had a history of atopy, with allergic rhinitis (75%) being the most frequent comorbidity. Median age at first reaction was significantly higher in the SRG (21 years old *vs* 14 years old; p = 0.026).

Regarding SPT results, there have been positivity for: LTP (n = 20; 100%), peach (n = 15; 75%), apple (n = 13; 65%), wheat (n = 10; 50%), hazelnut (n = 8; 40%), peanut (n = 7; 35%), *Parietaria* (n = 5; 25%), *Artemisia* (n = 5; 25%), plum (n = 4; 20%), tomato (n = 4; 20%), pear (n = 3; 15%), celery (n = 3; 15%), grape (n = 2; 10%), kiwi (n = 1; 5%) and plane tree (n = 1; 5%). The LTP molecular sensitization profile is shown in **table I**; Tri a 14 and Ole e7 have not been identified in our cohort, but sensitization to other panallergens has been identified: PR-10 in two patients (Bet v1, Fag s1, Mal d1, Cor a1, Api g1, Gly m4, Ara h8), both belonging to LRG, and profilin in one patient

Table I *- Epidemiological and clinical characterization of peach allergic patients' cohort.*

Total number of patients	**20 (n = 100)**
Age, years	28.8 ± 11.1 [18-68]
Gender, male/female	4 (20)/16 (80)
Age of first reaction to peach, years	18.2 ± 10.3 [1-48]
Allergy to >1 LTP food	18 (90)
Clinical manifestations	
Systemic reaction	13 (65)
Anaphylaxis	7 (54)
Urticaria/Angioedema	4 (31)
Gastrointestinal symptoms	2 (15)
Local reaction (Oral Allergy Syndrome)	7 (35)
Atopy	14 (70)
Rhinitis	15 (75)
Asthma	8 (40)
Eczema	5 (25)
LTP molecular components identified	
Pru p 3 (peach)	15; 8.7 ± 9
Mal d 3 (apple)	7; 1.9 ± 2.6
Act d 10 (kiwi)	2; 0.7 ± 1.3
Vit v 1 (grape)	4; 0.5 ± 0.8
Cor a 8 (hazelnut)	5; 1.1 ± 1.6
Ara h 9 (peanut)	11; 3.3 ± 4.2
Jug r 3 (walnut)	5; 0.7 ± 1.1
Sola l 6 (tomato)	1; 0.02 ± 0.03
Api g 2 (celery)	8; 0.3 ± 0.3
Zea m 14 (maize)	6; 0.6 ± 0.84
Par j 2 (parietaria)	4; 1.2 ± 1.9
Art v 3 (artemisia)	6; 0.4 ± 0.5
Pla a 3 (plane tree)	7; 1 ± 1.3
Can s 3 (hemp)	2; 0.78 ± 0.32

Data presented as n (%), mean ± SD and median (IQR) as appropriate; LTP: lipid transfer protein; SD: standard deviation.

(Bet v2) belonging to SRG. Also, gibberellin-regulated protein Pru p7 has not been identified in our sample.

sIgEmc mean value was significantly higher in the SRG: Pru p3 (12.71kUA/L *vs* 0.12kUA/L; p = 0.004), Mal d3 (2.99kUA/L *vs* 0kUA/L; p = 0.016), Ara h9 (5.01kUA/L *vs* 0.12kUA/L; p = 0.022) and Pla a3 (1.48kUA/L vs 0kUA/L; p = 0.037).

Regarding the molecular sensitization profile, a statistically significant association was found between the presence, individually, of sensitization to Pru p3, Mal d3 and Pla a3 and systemic reaction, as shown in **figure 1**.

Characterization of the molecular profile seems to be relevant as a marker of disease expression. Molecular allergology is increasing in clinical routine worldwide, which will help support the physicians' allergy workup (16, 17).

Also in our study, agreeing with previous ones, the most frequent clinical presentation was systemic reaction (65%) and Pru p3 was the most prevalent LTP (75%) (1-3).

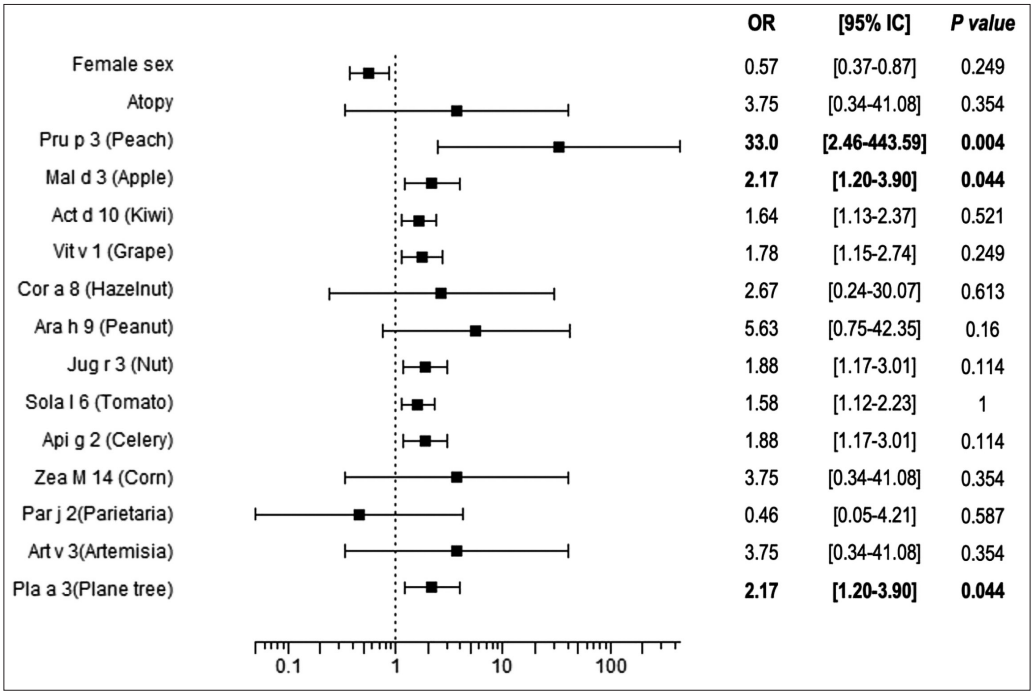
Although the presence of atopy was not significantly related to the severity of the reaction (p = 0.354), LTP pollens (*Artemisia*, *Parietaria* and plane tree) were also identified in our cohort, confirming the existence of cross-reactivity. In these patients, it may be equated the respiratory allergy as possible primary route of sensitization to LTP, according to the literature (3,15). However, the fact that we do not know which were the first symptoms to appear were (food *versus* respiratory) and the fact that sIgE values are higher for food allergens compared to respiratory allergens, may refute this theory.

As in other studies, it was verified that sIgEmc for, mainly Pru p3, but also Mal d3, Ara h9 and Pla a3, are directly related with the occurrence of systemic reaction and its severity (1, 2, 18).

The evaluation of agreement between symptoms, SPT and sIgEmc reached statistical significance only for hazelnut and celery (moderate agreement).

Study's limitations include the reduced sample, that could limit extrapolation of results, and the limited assessment of other peach molecular allergens besides Pru p3 and Pru p7, not available on ALEX®2, whose effects on symptoms were missed. Nevertheless, the authors believe that it has the value of being the first cross-sectional study worldwide characterizing peach allergic patients with ALEX®2, this new technique allowing to reduce the time spent by performing simultaneous IgE measurement of multiple total extracts and molecular components, helping in the identification of sensitization patterns associated with systemic reactions. These findings could improve patients' management in clinical practice, highlighting the importance of evaluating sensitization to other LTP proteins associated with Pru p3, instead of only Pru p3 itself.

***Figure 1** - Association between LTP proteins and the occurrence of systemic reaction.*



t-independent and Mann-Whitney tests were used to compare parametric and non-parametric independent samples, respectively, while Fisher's exact test or Qui-square test were employed to evaluate associations between categorical variables; P-values < 0.05 were considered statistically significant; OR: Odds Ratio; CI: Confidence interval.

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Contributions

MIS, MP, FD, MCPS, CC: conceptualization. MIS, RB, MP, MCPS: formal analysis. MIS, RB, MP, FCD, CC: data curation. MIS, RB, MP, FCD, MCPS, CC: writing – original draft. MIS, RB, MP, FCD, MCPS, CC: writing – review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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