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UN VIAGGIO LUNGO 45 ANNI: GUARDA IL VIDEO

A 45-YEAR JOURNEY: WATCH THE VIDEO

UN VIAJE 45 AÑOS DE DURACIÓN: MIRA EL VÍDEO

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SUMMARY

Notiziario Allergologico, 2025 Vol. 43, n. 2

Notiziario Allergologico

PDF VERSION

Notiziario Allergologico has been alive and well for over forty years. Today, it becomes international with a new layout that includes the translation of all content into three languages. The purpose remains unchanged if not implemented: to promote allergology culture by offering readers the possibility of an in-depth study and update on various allergology topics, also with a view to the future, thanks to the competence and authority of the authors of the articles published. The popular character of the articles contributes to making them accessible to a vast number of specialists, not only allergologists but also pulmonologists, paediatricians, dermatologists, etc.

ENGLISH

EDITORIAL

45 YEARS OF NOTIZIARIO ALLERGOLOGICO

Edited by **Gianni Mistrello**

The double birthday of Lofarma and the Notiziario Allergologico

In our culture, celebrations and festivities are particularly concentrated on the occasion of multiple repetitions of 10. In this case, a customary birthday takes on a very special character, since it coincides with the 80th anniversary of Lofarma, the Company to which we owe the birth of "Notiziario Allergologico", which instead crosses the finish line of its 45th year of publication and does so with a special issue that includes a selection of some of the most significant articles published during its forty-five years of existence, and which are testimony to the spirit that inspired its birth.

The brief introductions accompanying each piece are also an analysis of today's context in which these topics are still relevant and, in some cases, still at the centre of scientific and medical debate.

Brief history of the company

I am convinced that many readers would join us to toast the event, not only the main protagonists who are unfortunately no longer with us: first and foremost Dr. Centanni, one of the pioneers of Italian Allergology, who after the first successes shared with Eng. Tito Livio Vaglio (Administrator of the Lofarma Pharmaceutical Laboratory) the idea of specializing the Company (today Lofarma), privileging the field of the production of allergenic extracts to be used both in the diagnosis and therapy of allergic diseases. Thus, under his Direction, Lofarma began to develop, grow and become what it currently is: a Company that represents an important reality in the Italian and non-Italian allergology scene, always careful to propose products with a strong innovative impact on the market.

The history of Notiziario Allergologico

Returning to the Notiziario Allergologico, if Dr. Centanni was the inspiration, the great animator of the initiative that later led to the birth of the journal was Dr. Falagiani, the then Scientific Director. At that time Allergology was in its infancy but in great ferment compared to the past thanks to the efforts of many researchers, and Dr. Falagiani had the intuition to create an easy-to-consult tool that would help to spread it and make it usable by a growing number of specialists and non-specialists. The aim of the journal was to satisfy the curiosity and desire for greater knowledge of the various allergological aspects that were being acquired at that time, offering readers an opportunity for growth and cultural enrichment. Having this goal in mind, and thanks to his engaging enthusiasm, he was able to find concrete support in many experts in the field at the time who proved ready to collaborate, dispensing their expertise on the subject with continuous updates, always offered in popular terms precisely to facilitate understanding. Soon the journal became a point of reference in the allergology scene, and countless were the contributions of the various experts in the field who, by virtue of their expertise, contributed to its success. This is evidenced by the numerous appreciations received from many readers over the years, precisely because of the journal's scientific updating and popularization activities.

The first graphic version and format were really basic, but even then the contributions were of some relevance. Over time, both the graphics and format became more modern and appealing, while keeping the original spirit and identity of the journal intact. The contents were updated as they kept up with new scientific acquisitions, and the journal was enriched with new columns. It was precisely on the occasion of the inclusion of a new column (The Immunologist) that my involvement in the journal began, at the invitation of Dr. Falagiani. For years, I was responsible for writing short articles explaining in the simplest possible terms the various analytical and instrumental

The evolution of the graphic design over the course of 45 years since the foundation of the Notiziario Allergologico.

techniques that were being developed and applied to improve the standardization of allergenic extracts on which products for the diagnosis and treatment of allergic diseases were based. With the untimely death of Dr. Falagiani, the then President of Lofarma (Rubens Vaglio) invited me to pick up "the reins of the journal" with a commitment to preserve its mission. This is what I have attempted to do over the years, and the interest the journal continues to show among the medical profession constitutes for me and the editorial team (formerly Dr. Ottoboni, today Dr. Romagnoli) a source of pride and a strong incentive to continue this exciting experience.

In recent years, the advent of new technologies has inevitably led to a transformation in the ways of using information. Notiziario Allergologico, while maintaining its print version, has embraced this evolution with enthusiasm, adapting to the times without losing its identity. Today, in fact, the journal is also available online, offering readers, particularly younger ones, the possibility of consulting it in an even more convenient and immediate way. More recently, it has also been enriched with English and Spanish translations.

The decision to revamp the Notiziario Allergologico marked another step toward global dissemination of knowledge and greater exchange among professionals in the field. The introduction of the Lofarma Academy Column, edited by Dr. Franco Frati, is our concrete response to the need to cultivate scientific culture in new generations of physicians preparing to meet the future challenges of Allergology.

In addition to the memory of the protagonists, we now let the articles we have selected tell the story of the magazine.

I wish you a good read.

Gianni Mistrello, Editor of Notiziario Allergologico

For **Lofarma**, 2025 marks a double milestone

Edited by **Domitilla Vaglio**

As mentioned by Dr. Gianni Mistrello in the Editorial, this year we celebrate a double anniversary that marks a milestone in the history of Lofarma and Notiziario Allergologico. The magazine's 45th anniversary coincides with the 80th anniversary of our Company, a journey that, from the beginning, has been driven by a passion for Research, Innovation and Evolution in the medical-scientific field.

Lofarma has always believed in the importance of investing in Knowledge, Training and Continuing Education of the medical class in the field of Allergology. The Notiziario Allergologico, for more than four decades, has been a point of reference for all those physicians interested in updates on allergy-related issues.

With this double anniversary, therefore, we celebrate not only our long history, but also our continuing vision of the future.

I wish you a good read.

Domitilla Vaglio, President of Lofarma

ENG

Edited by **Gianni Mistrello**

Notiziario Allergologico
Year **I** • n. **11** • September **1980**

Both today's concept of specific immunotherapy traceable to the pioneering studies of Noon and Freeman (who treated subjects with certain clinical manifestations "in the dark" by administering aqueous pollen extracts only subcutaneously) and that of allergy originated at the beginning of the last century. The latter, in particular, thanks first to the Viennese physician Carl von Pirquet (he was the first to observe a change in an organism's ability to react to an antigen) and later to two German physicians, Prausnitz and Kustner (1921), with their demonstration that an allergic sensitization could be transferred via serum from an allergic subject to a healthy one. After these initial steps, no further significant progress was made on the subject for many years; progress that was extraordinary after the discovery of the existence of a new class of immunoglobulins by two Japanese researchers (Mr. and Mrs. Ishizaka), and that these immunoglobulins (now known as IgE) were present in the serum of allergic subjects.

In this very first work, which bears the signature of the prestigious Florentine research group of Prof. Romagnani's school (this was in 1980), it was found that lymphocyte cultures from the peripheral blood of subjects allergic to Gramineae pollen, when stimulated *in vitro* with Gramineae extract, produced specific IgE toward the allergens it contained, unlike what could be observed in lymphocyte cultures from healthy subjects. In the study, it was also observed that the production of specific IgE by certain specific cells was the result of previous activation of those cells that occurred *in vivo*. Needless to emphasize how after this first observation the Florentine research group assumed extraordinary relevance in the worldwide allergological scene, first with the demonstration of the existence also in humans (before then it was observed only in mice) of two subtypes of lymphocytes; one known as Th2 capable of producing cytokines (IL-4, IL-5 and IL-13) supportive of IgE production and consequently of allergic symptoms; the other known as Th1, capable of producing cytokines, such as IL-12 and interferon gamma, capable of blocking the effect of the Th2 subtype and thus inhibiting the allergic response. These observations have fuelled a worldwide series of research that has led to an extraordinary development in the understanding of the pathogenetic mechanisms of allergic diseases, as well as having important clinical implications in their treatment.

IN *VITRO* PRODUCTION OF IGE BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Romagnani S, Maggi E, Del Prete GF, Troncone R, Ricci M.
Department of Clinical Immunology, University of Florence.
Clin. **Exp. Immunol. 42, 167-174, 1980.**

This research group is at the forefront of the study of *in vitro* production of IgE, a most interesting experimental model for the study of allergy.
Indeed, it is well known that the production of IgE (as in general of all antibodies) is a very complex event, the result of various regulatory systems. There are cells that, although they do not participate directly in the immune phenomenon, play an adjuvant role in IgE production by acting on B-IgE lymphocytes: the T-helper lymphocytes. Other cells act on the same B-IgE lymphocytes by exerting an inhibitory role: T-suppressor lymphocytes.
These T lymphocytes act by means of soluble mediators that are different in chemical nature from immunoglobulins: studies are under way to determine to which subpopulations the various lymphocytes with different activity belong.
IgE production is thus the result not only of prior sensitization but also of the balance of these opposing forces. It is possible that there are other regulatory systems that we do not know about today, just as it is certain that the regulation known today is far more complex than described. These studies are important not only to increase our knowledge but also to lay the practical foundation for a new strategy of therapy of allergic diseases.
In fact, the goal of immunotherapy is to enhance those cellular components that have suppressive activity on IgE production: to achieve this, a thorough understanding of how these mechanisms work is essential. The study of IgE production *in vitro* could be a useful approach to this very important problem.
In this work, which is the first in a long and hopefully fruitful series of experimental publications, the authors detect total and grass-specific IgE levels in peripheral blood lymphocyte cultures of nonallergic subjects and grass-allergic patients cultured for 7 days: both sponta-

neous IgE production from unstimulated cells and from cells stimulated with polyclonal B-cell activators (pokeweed mitogen = PWM) were analyzed. Total (nonspecific) IgE was produced by cells from both normal and allergic subjects. In contrast, allergen-specific IgE was detected only in allergen cultures. The mean levels of total and specific IgE produced did not vary significantly depending on whether the cells were or were not stimulated with PWM.

The sequence of IgE production over time in comparison with the IgE content in the cells at the beginning of culture established the following:

1 - part of the total and specific IgE detected in the cultures after 7 days had not been produced *in vitro* but were found bound to membrane receptors on basophils and lymphocytes already at the beginning of culture;
2 - part of these IgE were already preformed and localized in cytoplasmic location: overall, preformed IgE constituted almost all IgE detected at the end of culture in most cultures of normal subjects and in some allergic patients;
3 - The majority of cell cultures of allergic patients instead synthesized IgE *in vitro*;
4 - Confirming this, it was observed that treatment of cells in culture with substances inhibiting protein synthesis (cyclohexamide and puromycin) inhibited the neoproduction of IgE;
5 - Treatment with mitomycin C, a substance inhibiting DNA synthesis and thus having antimitotic activity, also produced decrease in IgE present in the culture as early as day 3.

The authors conclude that spontaneous antibody production by IgE-producing cells in culture is the result of a previous activation that occurred *in vivo* in the organism: in fact, stimulation with a polyclonal B-lymphocyte activator (PWM) does not increase this production. Future research will seek to determine whether and how these IgE-producing cells can be influenced by *in vitro* manipulations.

* * * * *

edited by **Gianni Mistrello**

Notiziario Allergologico
December **1998** • Volume **17** • n. **4**

The dust mites of the genus *Dermatophagoides* that dominate our domestic environments unchallenged represent one of the main sources of environmental allergens, the inhalation of which, in sensitised and genetically predisposed individuals, can cause the appearance or aggravation of specific allergic pathologies. In the article dating back to 1998 (written by the undersigned together with Dr. Ottoboni, the expert acarologist at Lofarma), a review of the most relevant aspects was presented, starting with the discovery around the 1960s (thanks to three Dutch researchers, Voorhorst and the Spieksma-Boezeman couple) that the component of house dust responsible for the onset of a specific allergic pathology was to be attributed to the presence of particular mites (*Dermatophagoides*), which were relatively unknown until then.

Obviously, this discovery sparked off an innumerable series of studies that contributed over time to improving our knowledge of biology, food sources and the influence of certain domestic environmental factors (primarily humidity and temperature) that may favour mite growth. Subsequent studies then highlighted the importance of faecal particles as an allergenic factor, and in particular the role of the molecular component known today as Der p 1. In turn, these further observations laid the foundations for developing both environmental remediation measures (mattress covers, acaricides, etc.) capable of reducing the allergenic load in the home environment, and tests capable of detecting its presence in dust samples. These include the technological development of a 'do-it-yourself' version of a kit by means of which a patient can measure the Der p 1 concentration of the dust sample in his or her home by himself or herself, comparing the intensity of the spot colouration that may have developed to a reference colour scale. Based on the result, the patient can then assess the level of allergic risk to which he or she may be subject. Admittedly, the use of 'do-it-yourself' tests that allow direct visualisation without the aid of reading instruments has become familiar to many people today, given the impressive use of similar tests proposed in the Covid 19 period, but in those days only one 'do-it-yourself' kit existed: the pregnancy kit.

ENG

SYNTHETIC MAGAZINES

HOUSE DUST MITES: HISTORICAL BACKGROUND AND TECHNIQUES TO DETECT THEIR PRESENCE

GIANNI MISTRELLO - DANIELA RONCAROLO - FABRIZIO OTTOBONI

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SUMMARY

Allergy to house dust mites affects millions of people worldwide. The mites most frequently found in homes are those belonging to the genus Dermatophagoides (pteronyssinus and farinae); they can be considered cosmopolitan organisms because they are found wherever there are conditions of humidity, temperature and nutrition compatible with their survival. Certainly, some of our living habits in terms of 'home comfort' have contributed to a microclimate that is particularly favourable to mites. One of the most important mite allergens (known as Der p 1) is contained in faecal particles. It has been observed that levels of this allergen above 2 micrograms per gram of dust are associated with the risk of sensitisation and the triggering of acute symptoms in mite-allergic patients. To reduce if not eliminate this risk, environmental remediation measures should be implemented, which are more effective the more targeted they are.

It therefore becomes imperative to identify niches in the domestic environment that offer hospitality to these phylogenetically distant relatives of spiders.

In this article we review the different methods that can be used to detect the presence of mites in house dust, highlighting limitations and advantages for each. In particular, we present a 'do-it-yourself' kit recently developed in our Research Laboratories that, due to its ease of use and inexpensiveness combined with its sensitivity and specificity, seems to be particularly suitable for the purpose.

KEYWORDS

Mites – Dermatophagoides – Der p 1 – ELISA – Aclotest

NOT. ALLERGOL. 17: 128, 1998

House dust mite allergy is a disorder that affects millions of people worldwide. Recent estimates indicate that in Italy alone the phenomenon affects about 5% of the population and is constantly increasing.

Unlike other forms of allergy such as, for example, those caused by pollens, allergy to house dust mites is particularly debilitating because, as they live in the very same environment as the patients, they cause them a perennial symptomatology that can manifest itself as rhinitis and/or conjunctivitis in mild forms, up to bronchial asthma in more severe forms.

Recently, it has also been shown that exposure to house dust mites (figure 1) can also induce a pathological skin manifestation such as atopic dermatitis in sensitised patients.

One of the first reports of the probable role of mites as a cause of allergic diseases dates back to around the 1920s.

On that occasion, it was observed that a group of workers working in a grain warehouse developed an asthmatic disorder, the cause of which was identified as a mite (*Pediculoides ventricosus*) parasitising the grain. It was hypothesised that the inhalation of airborne particles, carrying dead mite fragments, was the cause of the disorder manifested by these workers.

This observation, along with others that followed, proved that food mites can cause allergic diseases.

Conversely, the importance of mites as a probable cause of allergy to so-called 'house dust' was only demonstrated

several years later, around the 1960s. Until then, allergists made the diagnosis, and consequently the therapy of allergy, using extracts obtained from house dust.
The study of house dust to discover the component responsible for allergic pathology interested several researchers. Suspicions were immediately focused in the direction of the organic component of dust. Thus, feathers from pillows, pet dander, fibre residues from textiles, tobacco, butterfly residues, wool, bacteria and moulds were some of the 'materials' considered. In no case was it possible to prove a correlation with house dust, leading to the conviction that house dust contained its own peculiar allergens, and the hypothesis was put forward that they were generated from the organic substances present there as a result of chemical degradation.
This theory was disproved around the 1960s when two Dutch researchers (Voorhorst and Spieksma-Boezeman) demonstrated unequivocally that the allergenic activity of house dust was of biological origin and identified mites of the genus *Dermatophagoides,* relatively unknown at the time, as the cause of house dust allergenicity. The observations of the two Dutch researchers triggered a series of studies that subsequently contributed to improving our knowledge of the biology, the food sources of house dust mites and the influence that certain environmental factors (humidity and temperature in particular) can exert on their growth. It has thus been ascertained that certain mites, such as those of the genus *Dermatophagoides pteronyssinus* and *farinae*, dominate home environments unchallenged because here they find favourable environmental conditions, such as a temperature of 20°-30°C and a relative air humidity of around 70%. This habitat amply justifies the cosmopolitan character of these mites; indeed, it is evident that such an environmental micro-climate is normally present in many homes and for several months of the year, especially at our latitudes.
Mites belong to the Arachnidae family (they are therefore related to spiders) and due to their size (200-400 thousandths of a millimetre) are invisible to the naked eye.
A female mite has a life span of three to five months, depending on the type of environment, and can lay up to 300 eggs. The mature adult individual develops in about three weeks after an initial larval phase. The mites' main biotope is the dust that forms on textile fibre materials in homes. This dust constitutes an excellent food source, thanks to the protein and glycidic contribution from the epithelial residues continuously eliminated by skin defoliation in humans. It has been calculated that man produces and releases about one gram of skin flakes into the environment daily, and this is enough for the survival and growth of thousands and thousands of mites. It has also been shown that the presence of moulds contributes to enhancing their growth because they may facilitate the elimination of the lipid coating of skin scales, performing a kind of predigestion that would make them particularly attractive to mites. The intake of food is carried out with the use of a mouthparts made up of chelicerae and the food then passes through the oesophagus, then the posterior middle intestine (where digestion takes place) to the distal part from where the excrement is emitted in the form of faecal 'pellets' that, due to their small size, can easily penetrate the airways.
One of the most important allergenic components of mites is concentrated in these 'faecal pellets', namely the allergen known as Der p 1, a glycoprotein with proteolytic activity that is probably responsible for its ability to induce allergy in humans.
Two micrograms of Der p 1 per gram of dust has been shown to be a threshold level for the risk of mite sensitisation. Apparently, there is no threshold value for mite sensitisation for asthmatic patients. This is precisely why it would be advisable for mite allergen levels to be kept as low as possible. The presence of mites in dwellings does not in itself denote a lack of cleanliness on the part of its inhabitants, but rather the existence of a natural symbiotic relationship, from which man thought he had freed himself by lulling himself into the illusion that soap and water and other achievements of civilisation were sufficient to avoid too much intimate contact with unwanted guests. In reality, certain habits of contemporary life (comfortable mattresses and sofas, upholstered furnishings, soft carpets, carpets, curtains, radiators, insulating windows...) while on the one hand they

Figure 1: **Enlarged specimen of a *Dermatophagoides* mite; the impressive buccal apparatus is visible in the foreground.**

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have contributed to making the domestic environment more and more comfortable, on the other hand they have created the ideal microclimate for the survival and development of house dust mites. Even if, given these premises, cohabitation with these beings seems inevitable, and constitutes the 'price to pay' for ensuring the 'comfort' of the home environment, it is worth remembering that today we have at our disposal a series of tools that can help us create difficult living conditions for mites, without necessarily making us give up our 'comfortable' habits.

The use of appropriate mattress and pillow covers made of materials that form an ultra-fine structure, and thus prevent the passage of mites from the mattress to the surrounding air environment, is already a first, simple environmental prophylaxis intervention that can help to significantly reduce the allergen load in our bedrooms, a favourite place for mites.

Another option, which falls within the scope of more specific preventive measures, is the use of products with acaricide activity. This treatment makes it possible to address the remediation of the home environment more broadly by limiting, if not eliminating altogether, the growth of mites in other areas of the home that may offer them hospitality, such as carpets, curtains, sofas, armchairs and other furnishings.

Several reliable products are currently on the market. Of these, those based on benzyl benzoate seem particularly effective.

It is clear, however, that environmental remediation is all the more effective the more targeted it is; this means that it is appropriate to identify those niches in the home that are infested by mites, thus exposing its inhabitants to the risk of sensitisation. With this in mind, it is undoubtedly useful to subject house dust taken directly from the home to an analysis in order to highlight the presence of mites and determine the concentration of their specific allergens

Among other things, this analysis is of considerable help in establishing the degree of effectiveness of the environmental remediation strategies implemented. Until a few years ago, the only technique available for detecting mites was microscopic observation of dust. While recognising the importance of this methodology, the application of which made it possible for the first time to demonstrate the presence of mites in dust and to specify their role as a potential cause of allergic diseases, it must be remembered that it is now outdated. Microscopic observation still remains a useful tool for the researcher who needs, for example, to recognise the predominant mite species in different geographical regions, or to study the biological characteristics of different mites, or the ratio between live and dead mites, or the differences between the adult and larval stages, but it has, conceived as a routine analysis of dust samples, a number of disadvantages that make it impractical.

The main disadvantages are:

- the preparation of the sample to be examined, as well as the evaluation under the microscope, are rather laborious and particularly time-consuming operations;
- the availability of appropriately trained personnel with basic knowledge is required, without which it is difficult to distinguish the characteristic elements of different species and sizes;
- does not allow the importance of the allergen load due to the presence of faecal particles to be determined.

One method for the determination of house dust mite allergens that is very simple and at the same time rapid, and therefore particularly suitable for routine application, is that based on the determination of the concentration of a marker, guanine. Guanine is contained in the droppings of arachnids in general, and is the final catabolite of purine metabolism. In house dust, excluding the presence of enough spiders to influence the test, mites thus appear to constitute the most representative arachnid species.

It follows that guanine determination can be considered a measure, albeit an indirect one, of mite allergens, as mite faecal particles are an important source of them.

The test result is the development of a characteristic colour due to the reaction between guanine and an aromatic diazonium compound. The intensity of the colour is directly proportional to the amount of guanine in the powder and thus to the amount of faecal particles. This intensity is assessed by comparing it with a colour scale that ranges from a reddish-orange colour (corresponding to a low level of infestation) to others with more or less intense red tones, which conversely indicate the presence of a considerable number of mites in the environment, with consequent risks of sensitisation or triggering allergic symptoms for those living there.

The method is semi-quantitative and can be performed by virtue of its simplicity even by non-experts because it does not require any equipment.

Unfortunately, the test has some limitations, in particular a certain inadequacy in terms of specificity, especially in the medium to low levels of positivity. Experiments conducted by various researchers have demonstrated the development of false positivity in dust samples that had tested negative for the presence of mites and their allergens, both by microscopic observation and by ELISA immuno-enzymatically specific for the allergen Der p 1, the latter being easily detectable, if present, in mite faecal particles.

It is therefore possible, even if the test company rules out both possibilities, that guanine from the droppings of other organisms (e.g. spiders or even birds) or other substances (e.g. uric acid, the final catabolite of insect nitrogen metabolism), interfering in the reaction, contribute to the development of the false positives mentioned above.

Another limitation of the test is that it does not allow the evaluation of the levels of other allergens, e.g. the one called Der p 2, which is absent in the particles as it is of somatic origin and instead present on the body of the mites themselves. The technological leap forward made a few years ago with the development of allergen-specific monoclonal antibodies made it possible to develop an enzyme immunoassay system

(ELISA) to assay the allergens (e.g. Der p 1 or 2, Der f 1 or 2) of mites present in house dust, which is still the most reliable method of assessment due to its remarkable qualities in terms of sensitivity and specificity.
It consists of the fixation of an appropriate amount of monoclonal antibody (e.g. anti-Der p 1) to the well of a microtiter plate, followed by incubation for a certain period of time with the powder sample (usually an aqueous extract obtained by adding an appropriate solution to the powder sample under examination). Subsequent incubation with a second monoclonal antibody (obviously directed towards a different determinant from the one recognised by the first antibody) conjugated with biotin, followed by the addition of the enzyme streptavidin-peroxidase and its substrate, results in the development of a colorimetric reaction whose intensity correlates with the amount of allergen present in the sample. This quantity is determined by interpolation on a reference scale obtained by adding known quantities of the same. Precisely on the basis of the use of the enzyme immunoassay method, an international group of researchers, a sort of Mite Task Force, among whom it is worth mentioning Professors Platt-Mills and Chapman (authors of numerous important publications on the subject) under the auspices of the WHO, has established a cause-effect relationship between exposure levels and 'health risk' for humans. In particular, this group, at the conclusion of their study, proposed that the presence in homes of Der p 1 levels above two micrograms per gram of dust should be considered an important risk factor for allergic sensitisation to house dust mites, while levels of 10 micrograms per gram constitute a risk factor for the development of acute symptoms for patients already sensitised to mites.
Although the enzyme immunoassay method is unanimously recognised as the most reliable for assessing mite allergen levels in house dust, it is not without limitations.
In fact, its execution requires the availability of trained personnel, the aid of laboratory equipment, including the reading instrument (spectrophotometer) and decidedly longer times (several hours) than the test based on guanine determination. In addition, and this is not entirely negligible, it is essential to subject the powder sample to an aqueous extraction because the analysis involves the use of a solution and cannot be performed using the powder directly. This therefore requires a certain amount of experience as well as

***Figure 2: Aclotest*, the do-it-yourself kit for detecting mites in household dust. The various components are visible: the test strip, the dispenser, the reaction tube containing the detector reagent in lyophilic form and the vial containing the solution to be used to dissolve the lyophil.**

***Figure 3: Aclotest:* test result in case of mites. You can see the coloured spot on the strip and the test reading scale in the background.**

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time in preparing the sample for analysis, an operation not contemplated in the test based on guanine determination.
Recently, our Research Laboratory, in an attempt to overcome the disadvantages and limitations associated with the use of the methods described above, while at the same time maintaining the sensitivity and specificity characteristics peculiar to the enzyme immunoassay, has developed an alternative method in the form of the development of a do-it-yourself version of a kit for detecting the presence of mites in house dust (figure 2).
The speed of execution, the direct use of the powder instead of aqueous extracts prepared from it, the direct visualisation of the result (without the aid of reading devices), combined with the simplicity of use, mean that the test can be easily performed and interpreted in meaning even by the patient himself.
The method (called *Aclotest*), which combines two different technologies (i.e. dot-blot and conjugation of proteins, in this case antibodies, with colloidal dyes) is based on the 'sandwich' use of polyclonal antibodies capable of recognising the different allergens of the mites *Dermatophagoides*, both *pteronyssinus* and *farinae*. These antibodies when fixed on the special membrane (dot-blot) placed on the test strip, act as a 'capturing agent' while acting as a 'revealing reagent' when conjugated to the colloidal dye.
The test is performed by placing the test strip in a test tube, into which the powder sample to be examined and the detector reagent had been added shortly before.
The aqueous solution in which the detector reagent is dispersed also performs an extraction function for any mite allergens present in the dust.
The appearance, after one hour of contact, of a pink-coloured spot on the test strip indicates the presence of mites in the sample examined. The different intensity of the spot colouration enables a distinction to be made (by relating it to a colour scale) between two quantitative levels corresponding to two different Der p 1 values (figure 3).
A less intense colouration indicates that Der p 1 values are between 0.5-2 micrograms per gram of dust levels (this still means the presence of mites even if at levels below the threshold associated with the risk of sensitisation), while a more intense colouration indicates the presence of mites with Der p 1 values well above the threshold and therefore with a high risk of inducing allergic pathology in the subject living in the place from which the dust sample examined was derived.
The absence of a signal, on the other hand, indicates the absence of mites or their presence but at negligible levels. This is related to the fact that the test has a sensitivity threshold that prevents the detection of levels below 0.5 microgram/per gram of dust.
The development of these methods of detecting aero-allergens of mites in the home environment may be of general interest to the population, but may become of paramount interest to asthma allergy patients and the physicians who treat them. Simple, easy-to-use and inexpensive tests such as the *Aclotest* make it possible to add a study of the home environment to the usual clinical assessment, and to identify those environments that, normally inhabited for several hours a day, are more likely to favour the development of respiratory allergy symptoms.
Likewise, they can provide objective means of assessing the effectiveness of environmental remediation measures, which when used in combination with specific immunotherapy, appear to be the winning strategy in treating mite allergy.

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Back in 2003, the date of writing this article, SLIT had recently been officially 'cleared' by international scientific societies through their respective consensus documents. The scientific literature of the time, although mainly exploratory in value, promised a flourishing development of this therapeutic tool. Only two years earlier, the European Community issued a directive (2001/83/EC) that framed Specific Immunotherapy with Allergens among allopathic medicines, thus forcing the need for robust evidence of efficacy, safety and immunologic activity, the latter imprinted to modify the natural evolution of allergic disease and provide that added value compared to conventional drugs, which is still the main goal of this treatment today. Subsequent literature has thus been largely enriched by confirmatory experimental evidence, and at the same time by observations in "*real-world practice*", the latter being essential to establish the real perceived benefit of immunotherapy, patient satisfaction, effect in the long term, and that therapeutic value useful to "*decision-makers*" in health policy.

Interestingly, however, there remained topics worthy of further investigation, such as those that the article identified as areas of needed investigation as early as 22 years ago. Namely, a better understanding of the mechanisms of action for different types of immunotherapies; dose-response relationships that cannot be generalized as they are strictly dependent on the formulations and characteristics of the extracts; characterization of the extracts in the absence of common analytical standards; effective preventive ability on the development of asthma in rhinitics; therapeutic adherence, which, especially for SLIT, can be a critical factor, but which can be improved through the implementation of "*Patient Support Programs*", the adoption of new electronic technologies, and the development of therapeutic products that allow flexible and widely tolerated use by the patient.

The continued spread of SLIT around the world, and the "appeal" exerted by it in emerging markets, make it possible to say that it still represents a modern therapeutic tool, on a par with the injection version, to be increasingly framed as an ally of the biologic world even for those patients suffering from severe pathology. Further research is highly desirable in this regard to enrich immunotherapy with added value in the health policy arena.

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SYNTHETIC MAGAZINES

SUBLINGUAL IMMUNOTHERAPY: STATE OF THE ART AND UPDATES

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SUMMARY

Sublingual immunotherapy (SLIT) is currently accepted as a viable alternative to the subcutaneous route in both adults and children, based on controlled clinical trials and post-marketing data. In the last two years, new studies have appeared that have clarified new aspects of the treatment. A meta-analysis study confirmed the clinical efficacy of SLIT in rhinitis, giving it evidence category 1A. At the same time, efficacy was also demonstrated in allergic conjunctivitis and SLIT was shown to reduce IL-13, thus promoting the TH2-TH1 shift. For the first time, it was formally confirmed that SLIT has long-term efficacy, like injection therapy, and that it can prevent the onset of new sensitisation. Compliance has always been considered a critical point of SLIT, but recent data have shown that adherence is actually excellent. In conclusion, the data supporting SLIT are constantly increasing and new aspects are being clarified. However, a number of question marks remain, including the problem of optimal dosing, the problem of preventing asthma in rhinitic subjects, and the problem of clearly defining mechanisms. These are likely to be the guidelines for future studies.

KEYWORDS

Sublingual immunotherapy – Asthma – Rhinitis – Safety – Adherence

1. Introduction and historical background

Specific immunotherapy (AIT) or allergen-specific vaccination is understood as the administration of gradually increasing doses of allergen, up to a maintenance dose, which is then administered at regular intervals. The purpose of AIT is to desensitise the subject to subsequent contact with the allergen itself, so that symptoms are reduced (1). For historical reasons, AIT has always been administered subcutaneously, although anecdotal reports of oral or bronchial administration are not lacking (2, 3). In 1986, the first official reports of serious (some fatal) side effects due to AIT appeared (4). These reports represented a major boost to the study of non-injection routes (oral, sublingual, intranasal, bronchial). The sublingual route (SLIT) immediately appeared very promising and, in fact, in the following years the oral and bronchial routes were progressively abandoned in favour of SLIT (3). The history of SLIT in official documents is summarised in Table 1 (1, 5-7).

Interestingly, in the course of only 10 years, the international scientific community has accepted SLIT as an alternative to subcutaneous, for use in routine clinical practice (7). This fact is the result of a large number of randomised controlled clinical trials, post-marketing surveillance studies, as well as results of laboratory and pharmacokinetic studies. In this article, we will not deal in detail with all clinical efficacy and safety studies, for which extensive reviews are available to which we refer (2, 3, 8), but we will examine the most recent results on the subject and future directions.

2. Clinical effectiveness

The clinical efficacy of SLIT, understood as a reduction in symptoms and drug consumption, is well documented in 25 randomised controlled trials (table 2). Of these, only three yielded doubtful or negative results (9-11), and this percentage is very similar to that observed for the injection route (12). As far as allergic rhinitis is concerned, a recent meta-analysis by the Cochrane Collaboration (13) confirmed by wide margins that SLIT is clearly more effective than placebo and therefore the

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Table 1. **SLIT in official documents**

Document	Year	Judgement
EAACI Position Paper	1993	'Promising... More data needed... Risk of too rapid absorption of the allergen'
BSACI Position Paper	1993	NOT CONSIDERED
WHO Position Paper	1998	'Valid alternative to subcutaneous AIT in the adult... More safety data is needed in the child...'
EAACI Position Paper	1998	IDEM
ARIA document	2001	"It can be administered in common clinical practice in both adults and children..."

Table 2. **Randomised controlled SLIT studies (for detailed bibliography see bibliography entry 6)**

Author- year	Range- Age	Patients A/ P *	Allergen	Duration	Cumulative Dose	PatoL **
Tari, 1990	5-12	30/28	Mites	18 months	365 STU	R/A
Sabbah, 1994	13-51	19/29	Grasses	17 weeks	4,500 IR	R
Feliziani, 1995	14-48	18/16	Grasses	3.5 months	25 BU	R
Troise, 1995	17-60	15/16	Parietaria	10 months	105 BU	R
Hirsch, 1997	6-16	15/15	Acan	1 year	570 mg Der p 1	R/A
Passalacqua, 1998	15-46	10/9	Mites (monoid)	2 years	10,000 AU	R
Vourdas, 1998	7-17	33/31	Olive	2 years	4 mg Ole e 1	R/A
Clavel, 1998	8-55	62/28	Grasses	6 months	28 mcg Phl p 5	R/A
Horak, 1998	16-48	18/16	Birch	4 months	250 STU	R
Hordijk, 1998	18-45	30/27	Grasses	6 months	300,000 BU	R/A
Bousquet, 1999	15-37	15/15	Mites	2 year	104,000 IR 4.2 mg Der p 1	A
Passalacqua, 1999	15-42	15/15	Parietaria	8 months	265 BU 16 mcg Par j 1	R/A
Pradalier, 1999	6-25	59/61	Grasses	4 months	11,000 IR 0.9 mg Phi p 5	R/A
La Rosa, 1999	6-14	20/21	Parietaria	6 months	75,000 IR 52 mg Par j 1	R/A
Purello, 1999	14-50	14/16	Parietaria	8 months	200 BU 12 mcg Par j 1	R/A
Pajno, 2000	8-15	12/12	Mites	2 years	360 mcg Der p 1A	
Guez, 2000	6-51	24/18	Mites	2 years	90,000 IR 2.2 mg Der p 1	R
Caffarelli, 2000	4-14	24/20	Grasses	3 months	37,000 AU	R/A
Ariano, 2001	19-50	10/10	Cypress	8 months	250,000 RU	R/A
Bahcecilier, 2001	7-15	8/7	Mites	6 months	7,000 IR 0.56 mg Der p 1	R/A
Voltolini, 2001	15-52	24/13	Mixed trees	24 months	250,000 RU 0.9 mg Phl p 5/ months	R
Lima, 2002	16-48	24/22	Grasses	18 months	90,000 IR 2.2 mg Der p 1	R
Mortemousque, 2003	6-60	26/19	Mites	24 months	variable	C
Andrè, 2003	6-55	48/51	Ambrosia	7 months	57 mcg Der p 1	R
Ippoliti, 2003	5-12	47/39	Mites	6 months	7,000 IR 0.56 mg Der p 1	R/A

*A/P = Active/Placebo **R=Rhinitis A=Asthma C=Conjunctivitis

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experimental evidence is to be classified as IA (resulting from numerous randomised controlled trials). The studies concerning asthma are fewer in number and therefore do not yet allow for a reliable meta-analysis, but the results reported in clinical trials are for the time being positive studies (14-18). Recently, a randomised controlled trial was published that even confirmed the clinical efficacy of SLIT in isolated mite allergic conjunctivitis (19). It should be noted that an equivalent study has never been conducted with injection AIT.
The most recent study available (20) conducted on 99 patients allergic to *Ambrosia* confirmed the earlier findings, formally indicating that the efficacy is at least partly dose-dependent. The comparison between SLIT and injection AIT has always been one of the most controversial points of the issue, and there are in fact a few studies on the subject (21-24). However, it must be considered that an objective comparison between two different methods of administration can only provide reliable answers if it is conducted in a double-blind, double-dummy fashion. At the moment, only two studies are available which correspond to the above-mentioned criterion. The first (25) was conducted on 20 grass-allergic patients and confirmed that there were no significant differences between the two treatments with regard to clinical efficacy, although this study lacked a placebo control group. The second double dummy study, currently being published (26) also includes the placebo group and shows, in a population of birch-allergic patients, that SLIT and subcutaneous AIT are statistically equivalent and superior to placebo. The improved safety profile of SLIT is emphasised in this study.

3. Security

Subcutaneous AIT is known to carry a risk of severe or fatal systemic reactions, as documented extensively in the literature. This risk can be reduced by carefully following safety recommendations, but cannot be eliminated (27, 28). On the contrary, no serious or life-threatening adverse events and no cases of anaphylaxis were ever reported during 15 years of clinical trials with local routes. With SLIT, the most frequent side effects are gastrointestinal (oral pruritus, nausea, abdominal pain), which are transient and can be easily controlled by dosage reduction. On the other hand, no serious systemic side effects have ever been reported and the incidence of systemic effects is the same in treated and placebo patients and also in adults compared to children (29). Certainly, controlled clinical studies provide rigorous data on safety, but more complete and reliable information comes from post-marketing surveillance studies, which analyse the side effects reported by patients in a situation that is more in line with reality. There are two such studies, one paediatric (30) and one adult (31). Both confirm that the total incidence of reported side effects does not exceed 10% of patients. Virtually all adverse events are minor and never lead to discontinuation of treatment. It is noteworthy that no change in mast cell mediators at the sublingual level is observed in children, even in the case of reported local symptoms (32). Finally, sublingual AIT seems to cause no local side effects even in patients with oral allergy syndrome (33).

4. Mechanisms of action and pharmacokinetics

The study of the mechanisms of action of non-injection AITs is only just beginning, as the primary objectives of the studies to date have been essentially clinical. From the point of view of basic immunology, SLIT (34) was shown in an ex vivo in vitro study to reduce the proliferation of allergen-specific T-lymphocyte clones. In vivo, sublingual AIT was observed to reduce the expression of the adhesion molecule CAM-I on the nasal and conjunctival epithelium and the concomitant infiltration of inflammatory cells (35, 36). A recent open-label controlled study in 10 children confirmed the reduction of the ICAM-1 molecule on the nasal epithelium, in association with a reduced response to methacholine (37). A paediatric study by Ippoliti et al. also showed a reduction in plasma IL-13 levels, confirming the possible effect of SLIT on the TH1/TH2 lymphocyte balance (38). The effect on non-specific bronchoreactivity may be considered as indirect evidence of an anti-inflammatory effect. Indeed, the non-specific bronchoreactivity observed in allergic asthmatics is at least in part sustained by bronchial inflammation. In this regard, in addition to the already mentioned study by Silvestri et al. (37), there is another open-label one conducted on grass-allergic patients where a significant reduction in the response to methacholine was demonstrated after three years of seasonal SLIT (39). More recently, a randomised, double-blind study confirmed in paediatric patients that SLIT for *Parietaria* is able to abolish the increase in non-specific reactivity during the pollen season (40). With regard to the pharmacokinetics of allergens, it is interesting to note that no data exist on the absorption of subcutaneous AIT despite almost a century of clinical use. For the mucosal pathways, some, albeit controversial, data already existed in animals (41).
Very recently, pharmacokinetic studies have been conducted in humans, using a purified radiolabelled allergen and assessing its absorption and progression by scintigraphy and plasma chromatography (42, 43). It was observed that:

a) there is no direct sublingual absorption;
b) part of the radiolabelled allergen persists for a long time attached to the nasal and buccal mucous membranes;
c) the modified allergen (monomeric allergoid) can be absorbed intact into the circulation (43).

The fact that the pure oral route (immediately swallowed allergen) is not effective, while the sublingual route is, and the persistence of the allergen at the mucosal level suggest that contact with the mucosa is a critical phase for the mechanism of action.

5. Controversial aspects and future prospects

One of the still unclear aspects of SLIT is that of optimal dosing. In fact, a review of the published studies only suggests that SLIT is effective at doses 3 to 400 times higher than those used in subcutaneous AIT, but it is still unclear what the best dosage is. On the other hand, it is true that as the dosage increases, the side effects increase, at least the gastrointestinal ones (44). A recent study by Andrè et al. (45) has shown, however, that there is a certain direct relationship between dose and clinical effects (the higher the dose, the more obvious the improvement in symptoms). One of the arguments to the potential detriment of SLIT concerns adherence to treatment (compliance), since the vaccine is administered directly by the patient at home. Actually, with the subcutaneous route (which should theoretically 100% adherence), the discontinuation rate due to the occurrence of side effects is very high and thus adherence is far from optimal (46, 47). Given the excellent tolerability of SLIT, good compliance is therefore expected. This problem was recently addressed in a multicentre study specifically dedicated to the quantitative measurement of adherence, and carried out with monomeric allergoid tablets (Lais, Lofarma). Patients were contacted by telephone unannounced and asked to count the tablets remaining in the blister pack. A computerised programme, based on the start date of therapy, counted the expected remaining number of tablets and thus the missed doses. Data are currently available for 35 patients and adherence is over 95% (48), regardless of allergen type and administration schedule. Again, with a view to improving adherence and making it easier for patients to take the therapy, one future prospect is to use a single dose from the start of treatment, avoiding the phase of gradual increase. This is again enabled by the good safety profile of SLIT. Recent work with maintenance dosing from the start confirmed this possibility: in 4 groups of patients treated with 3 different dosages, one of which was high from the start, no significant changes in the incidence of side effects were observed (49). It is well known that injection AIT is able to maintain clinical effects even for years after discontinuation of treatment (50). Recently, this peculiar property was also demonstrated for SLIT in a 10-year prospective study of 60 asthmatic children treated with SLIT for dust mites (51).

Certainly, this important observation requires further confirmation, and thus the issue of the long-lasting effect will certainly be the subject of study in the years to come.

Certainly, the preventive effect on the onset of asthma in rhinitic patients, which is well known for the injection route, remains to be proven for SLIT (52). It has also recently been reported that SLIT can also prevent the onset of new skin sensitisation (53): 5.9% in patients treated for three years versus 35% in the control group.

6. Conclusions

SLIT is still widely used in many European countries and its routine use has been validated in the most recent official documents. Its indications do not actually differ from those for the injection route and the same recommendations for prescription apply. The latter should always be carried out by the specialist, after accurately establishing the diagnosis, assessing the cost/benefit ratio, and after informing and educating the patient on how to use it and the possible side effects. After an initial period of scepticism, the international scientific community is slowly becoming aware of the real therapeutic possibilities of SLIT (3, 54), even if contrary or critical opinions remain (55). It is also true that some important points remain to be clarified: the optimal dosage, the mechanisms of action and the preventive capacity against asthma. These aspects will certainly be the subject of future clinical and basic immunological studies.

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Allergenic extracts (allergens) used for both diagnostic and therapeutic purposes have been ignored for very many years by the body of law in health care; their registration journey began, at least in Italy, in 1991 with the recognition that these products fully fall within the definition of medicinal products. In this article, Dr. Falagiani (Scientific Director of Lofarma until his untimely death), after reviewing the regulations that generally govern the registration of medicinal products in Italy and Europe, focuses his attention on the regulatory aspects of Allergens, a subject (this is 2004) little known even to the most experienced experts in the field. The story begins with the then Ministry of Health, which, taking note of the existing situation, attempted to regulate these particular medicines, promulgating a decree (Ministerial Decree December 13, 1991) by which it allowed Manufacturing Companies to continue to produce and market them, provided that they were produced before October 1, 1991, and provided that they submitted to the aforementioned Ministry a regular application for an A.I.C. (marketing authorization), required the submission of a very substantial dossier. This particular treatment (at least with respect to therapies) by the Ministry, which differed from the registration route required of normal medicines, was also justified by the fact that therapies were produced on the basis of an individual prescription for a given patient (*named patient*). As mentioned by the author, however, this did not prevent the aforementioned Companies from being regularly inspected by the Health Authorities for compliance with Good Manufacturing Practice (GMP) standards for the medicines produced and quality control of the same, in accordance with the *Note for Guidance on Allergen Products* guideline at the time. Obviously, this guideline is continuously updated going hand in hand with technological progress, and the relevant dossier is adapted to it from time to time. On the issue of the issuance of the A.I.C., it is worth mentioning that even today the hopes of Companies to have their expectations fulfilled are lacking. Following the latest Determination (DG 2130/201) AIFA (which has since taken over from the Ministry of Health in the management of the problem) has requested additional fulfilments from the Manufacturing Companies. Based on an initial assessment, AIFA allowed the continued *ope legis* marketing of products that met the requirements. On the other hand, with regard to the issuance of the A.I.C. of the same, we are waiting for the conclusion of the transitional phase. To date, however, AIFA specifies that in Italy the typology of allergen medicines on the market is as follows:

a) Medicines authorized for marketing ope legis under Ministerial Decree Dec. 13, 1991;
b) Medicines with regular A.I.C. (covers only some Allergens);
c) Medicines marketed under Article 5 of Legislative Decree 219/2006 as *Named Patient* Product (NPP).

REGULATORY ASPECTS OF ALLERGENIC PRODUCTS

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SUMMARY

The main regulations governing the registration of medicinal products in Italy and Europe are reviewed. More specifically, the structure of the registration dossier according to the Notice to Applicants format is described.

A process of harmonisation of the requirements for medicinal products and control procedures for registration, known as ICH, is underway, which will lead to unification between Europe, Japan and the USA. The position of allergenic products in Italy, before and after the 1991 decrees, and in the various European countries, and their particularities that differentiate them from normal medicinal specialities are examined.

As for the quality of allergy products, it is defined by recent European Guidelines, Note for Guidance on Allergen Products and Pharmacopoeia.

Finally, we examine the regulatory situation for allergen products in the US, which differs substantially from Europe in that intermediate products are registered there, and the preparation of immunotherapies is carried out directly by allergists.

In conclusion, it is hoped that the dual channel of production and distribution of registered and individually prepared products will be confirmed for these products in order to meet the present and future therapeutic needs of allergists.

KEYWORDS

Allergenic products - Marketing authorisation (registration) - Decrees - Guidelines - Pharmacopoeia - ICH.

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1. Introduction

Allergens are very special products compared to normal medicines. Foremost, they have a characteristic that makes them unique. They have immunological, and therefore in a broad sense 'pharmacological', activity only in subjects with allergic sensitisation, whereas for normal subjects they can be assimilated to normal proteins. It is therefore natural that their toxicity, when studied using normal tests on non-sensitised laboratory animals, is practically nothing, so much so that the Pharmacopoeia, as we shall see later, only requires them for mould extracts intended for parenteral administration.

Another characteristic that makes them very different from normal drugs is the complexity of their chemical structure. Indeed, since they are obtained by extraction from complex allergenic sources (pollens, mites, fungal spores, animal epithelia, etc.) they contain a large quantity of proteins, small peptides and pigments. With special purification techniques, it is possible to remove most of the small peptides and pigments, which have no immunological effectiveness. What remains is a very heterogeneous protein mixture. Suffice it to say, by way of example, that a pollen extract of *Phleum pratense* contains 9 different known allergens, that of *Dermatophagoides pteronyssinus* 11, that of cat epithelia 7, that of Alternaria alternata 9. This heterogeneity also explains the complexity of the analytical processes and standardisation of these products.

In order to better frame the specific regulatory position of allergen products, some background on drug registration is appropriate.

2. Drug registration in Italy and Europe (1)

2.1 Italy until 1991

Pharmaceutical legislation in Italy was for more th an fifty years regulated by the Royal Decree of March 1927, later supplemented by the *Testo Unico delle Leggi Sanitarie* of July 1934. These laws established the obligation to obtain authorisation from the Ministry of Health both to produce and to market pharmaceutical products. They also stipulated which documents had to be provided to obtain authorisation: in essence, it was necessary to prove quality, safety and efficacy.

2.2 Europe

The year 1965 saw the start of a long legislative journey on medicines at European level, with EEC 65/65 being the first directive to establish criteria for the acceptance of medicines by member states. It affirms the fundamental principle that must govern the granting of marketing authorisation for medicinal products: it must be based exclusively on technical criteria (demonstration of quality, safety and efficacy) and not on political, economic or any national expediency. Subsequent directives have been issued, with the

aim both of improving the level of pharmaceutical production but also, and above all, to harmonise the policies of the Member States. The figure of the 'qualified person' was created, who within the pharmaceutical company represents the person responsible for the quality of the product and who acts as its guarantor, eliminating the need for rechecks at the time of importation into other Member States (Directive 75/319). Community registration procedures such as the 'multi-state procedure' were established, which consists of the possibility of mutual recognition by other European Community (EC) countries on the basis of the same documentation (dossier) submitted for the first authorisation, supplemented by an assessment report (Directives 75/319 and 83/570). A supranational regulatory structure, composed of experts from national EC regulatory structures, called the CPMP (*Committee for Proprietary Medicinal Products*), was created to manage the multi-state procedure and is still the EC's pharmaceutical advisory body today (Directive 75/319).

2.3 The dossier according to Notice to Applicants

Also, in the vein of harmonisation between the various EC Member States, the creation of a standardised template for the preparation of registration dossiers, known as '*Notice to Applicants*' (NTA) (Directive 83/570), was of great importance.

The file according to Notice to Applicants is composed as follows:

Part I: Contains the administrative data of the pharmaceutical company applying for the Marketing Authorisation (MA) and the three Expert Reports relating to each of the subsequent parts.

Part II: Contains information on the chemical nature of the drug, its manufacturing process (and its validations), quality control, all in compliance with Good Manufacturing Practices (GMP).

Part III: contains pharmacological and pharmacokinetic information and the results of toxicological tests in animals, which must be carried out in accordance with Good Laboratory Practices (GLP).

Part IV: Contains the results of clinical trials to ascertain the efficacy and safety of the drug. They must be performed according to Good Clinical Practices (GCP), already drawn up in 1977 under the name Proposed Regulation by the Federal Register of the Food and Drug Administration (FDA) in the USA, and made mandatory in Europe in 1991 (Directive 91/507) and in Italy in 1992 (OM 27.04.1992).

According to GCP, clinical studies must observe the following criteria:

I **Ethicality**: the need to obtain authorisation from the relevant Ethics Committee and informed consent from participants, as well as to ensure their protection throughout the study;

II **Responsibilities**: clear indication of the obligations of the sponsor, of the doctors performing the study, of the monitors verifying its progress;

III **Data management**: indication of the criteria for collecting, processing and storing data, while respecting privacy;

IV **Statistics**: the statistical methods that will be used to process the results should be indicated, specifying the criteria that led to the study design and the number of patients to be enrolled;

V **Quality**: the quality assurance system for the entire management of the study data should be indicated.

The registration dossier is the heart of Marketing Authorisation Application (MAA, commonly referred to as registration), which can be submitted to a Marketing Authorisation Authority to obtain authorisation in a single country according to the national procedure or to the European Medicines Evaluation Agency (EMEA) to obtain European authorisation according to the centralised procedure (Regulation EEC 2309/93). The centralised procedure under the EMEA is the only one allowed for high-tech drugs, e.g. those obtained by recombinant DNA technique or monoclonal antibodies or their derivatives. Since July 2003, the structure of the registration dossier must follow a new format, called Common Technical Document (CTD), which entails substantial formal changes compared to the Notice to Applicants.

2.4 The ICH harmonisation process

At present, the major geographical areas of the world have their own laws on inspections of pharmaceutical production sites and registration of medicines. This means that pharmaceutical companies with global markets have to undergo inspections by all states where they want to import their products and make separate registrations, with often different dossiers. For example, a European company that wants to export a medicine to the US must apply for (and pay for) an inspection by the FDA to verify that the medicine is produced according to US manufacturing regulations (GMP). In addition, it will have to apply for marketing authorisation from the FDA by submitting a dossier that complies with US regulations. If,

for example, you also want to take your medicine to Japan, you will have to repeat the same procedure with that country. If there are then any discrepancies between the regulations of one country and those of another, conflicts will obviously arise. Furthermore, this situation leads to a very heavy increase in registration costs. To give an idea of the financial dimensions of research and development in the pharmaceutical field, suffice it to say that the average international cost of developing a new medicine, from the start of research to the pharmacy counter (including molecules that abort for various reasons), is today around USD 830 million.

In order to harmonise regulations and procedures, and thus foster a globalisation of markets, a collaborative project between Europe, Japan and the USA was initiated. This project envisages, through an ongoing dialogue between authorities and pharmaceutical industries, to identify guidelines and requirements acceptable to the three parties in dialogue. This process, which has the name '*International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*', abbreviated to the acronym ICH, began in 1991 with a conference between the three parties, which was then repeated every two years. In this project, several expert committees (ICH Steering Committees) were set up and drew up guidelines, which were immediately implemented by the individual states. As an example, we can mention the Guideline on Stability Testing of New Drug Substances and Products, which establishes what the storage conditions must be (temperature, humidity, time), what the criteria for selecting batches for analysis should be, what the criteria for assessing degradation and residual activity should be, and consequently what information should be stated on the label. For example, a medicinal product that does not require special temperature conditions for transport and storage (e.g. 2-8°C) must have passed unscathed accelerated stability in an environment of 40°C± 2°C with relative humidity (RH) of 75%±5%. Only if it complies with this ICH Guideline is the stability of medicinal products accepted for authorisation by all regulatory bodies. Naturally, the ICH area of influence will quickly extend to other geographical areas, eventually creating an almost worldwide harmonisation.

2.5 The Italian DL No. 178 (2)

In Italy, the only health laws in force until 1991 were the aforementioned Royal Decree of March 1927 and the Consolidation Act of July 1934, even though the law stipulated that it was mandatory for each Member State to comply with the European Directives in any case. On 19 May 1991, Legislative Decree (DL) No. 178 entitled 'Transposition of European Economic Community Directives on medicinal products' was promulgated in Italy, transposing European Directives 65/65, 75/319 and 83/570. It reorganises all the rules on the authorisation for the production, importation and marketing of medicinal specialities, extends the responsibilities of the technical director of the manufacturing company, and tightens the administrative, pecuniary and criminal sanctions for those who violate these rules. Decree-Law No. 178, in Article 25.4, provides for the possibility that unauthorised medicinal products may be produced and marketed 'at the doctor's request, in writing and unsolicited, who undertakes to use the products on their own patients or those of the facility to which he is assigned, under their direct and personal responsibility'. In addition, DL No 178 issues in Article 20 special provisions for certain medicinal products, including allergens, which are defined for the first time, as we shall see later (3.2).

3. The regulatory status of allergens in Italy

3.1 The situation up to 1991

Until 1991 (the year DL No 178 came into force), allergens in Italy had been completely ignored by the body of health law. However, many companies operated, and many still operate, producing allergens, i.e. importing them from foreign parent companies. These companies have always been, and still are, regularly inspected by the Ministry of Health (now the Ministry of Health) for compliance with Good Manufacturing Practice (GMP) in both production and quality control of allergens. In favourable cases, these inspection visits resulted in decrees authorising the production of allergens. We are thus faced with an unusual situation, in which the production of a drug is authorised, but the drug itself does not have its own specific marketing authorisation (MA). The explanation for this lies in the fact that while allergens are a special category of drugs, they cannot but be considered 'medicinal products', they are not 'medicinal specialities'. Let us analyse the definitions of 'medicinal product' and 'medicinal speciality' (as per DL No 178).

Definition of medicinal product: '*Any substance or combination of substances presented as having properties for treating or preventing disease in human beings or animals, as well as any sub-*

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stance or combination of substances to be administered to human beings or animals with a view to making a medical diagnosis or to restoring, correcting or modifying organic functions in human beings or animals'. As can be seen, allergens, whether for *in vivo* diagnosis (e.g. prick tests) or immunotherapy, fall fully within this definition.

Definition of proprietary medicinal product: '*Proprietary medicinal products are those medicinal products previously prepared and placed on the market under a special name and in special packaging*.' Allergens produced in Italy do not fall under this definition. In fact, immunotherapies are always personalised with the patient's name (named-patient), even when they contain a standard allergen (e.g. grass pollen mix or *Dermatophagoides*), and especially mixtures of two or more allergens that are prepared on prescription. This is not just a formal difference, but a substantial one. In fact, the customisation of vaccines entails active surveillance by the company, with assumption of responsibility, on the correctness of prescriptions (e.g. a request for maintenance therapy for a patient who never ordered the initial therapy is intercepted and reported to the doctor), product traceability, etc. A completely different pathway from medicinal products, of which the manufacturing company loses all trace once delivery to the pharmaceutical wholesaler has taken place. This particular status of allergy products has justified special treatment by the authorities.

Figure 1: Paul-Ehrlich-Institut until the 1980s (left) and current location (right) (credits to pei.de)

3.2 Allergens in DL No 178

As previously reported, DL No 178 was promulgated on 29 May 1991, where allergens were mentioned for the first time. It is worth reading literally what is stated in Art. 20.2: '*Allergens are also subject to the regulations of this decree, meaning medicines that are intended to detect or induce a specific acquired alteration in the immunological response to an allergenic agent*'. One may be puzzled by this definition. It is unclear, in fact, why allergens should '*induce a certain acquired alteration*...'. If the author had been referring to immunotherapy allergens, the correct term would have been '*modify a certain acquired alteration*...'. In any case, this definition was not created by the Italian legislator, but is the translation of the definition in the European Directives.

It is important to note that DL No 178 defines two particular categories of medicines, allergens, as mentioned above, and radiopharmaceuticals. The latter are defined in Art. 21: '*For the purposes of this decree, a radiopharmaceutical is defined as: any medicinal product which, when ready for use, includes one or more radionuclides (radioactive isotopes) incorporated for health purposes*'. What did allergens and radiopharmaceuticals have in common? Basically, the fact that they have very special characteristics that make them different from other medicines. This decree therefore recognises that the technical peculiarities of these two categories of medicinal products, although very different from each other, entail the need for separate treatment also at the regulatory and, as we shall now see, regulatory level.

3.3 Allergens in the Decree 13.12.1991

A few months after DL No 178 came into force, precisely on 13.12.1991, the Ministry of Health issued a decree specifically dedicated to these two particular categories of drugs, entitled '*Provisions on radiopharmaceuticals and allergens*' (3). This decree represents a kind of 'amnesty' for the two categories of drugs that had been ignored by previous legislation. It is important to note that the Decree of 13.12.1991, which expressly refers to DL No 178, states three very important concepts in its introductory part:

I an indication of the '*advisability of providing companies that produce and market allergens and radiopharmaceuticals with instructions aimed at ensuring that the production and trade of these products is brought into line with the regulations of the aforementioned legislative decree*';

II the acknowledgement that '*several authorisations for the production of allergens and radiopharmaceuticals*

have been issued by this Ministry';

III the statement that '*in order to avoid the risks associated with the unavailability of medicines deemed necessary, the further sale and use of radiopharmaceuticals and allergens should be permitted, subject to certain conditions, pending the determinations that will be made on applications for marketing authorisations for the same products*'.

In essence, Decree of 13.12.1991 asks manufacturers to indicate which products were already in production and trade on 1 October 1991.991, and only of these does it allow for continued production and trade beyond 1 January 1992. This is therefore an acknowledgement of the existing situation and a relative amnesty. Furthermore, this decree required companies to submit dossiers describing the production and quality control of allergenic products, one for each of the 13 allergen families (plant and animal foods, grass pollens, grasses and trees, mites, mycophytes, insects, epithelial derivatives, environmental dusts, hymenoptera venom, bacteria, allergens of chemical origin), by 30 April 1992. For the pharmacotoxicological and clinical parts, the Decree stipulates that they may be prepared in a common way, for the various types of products, by all the companies concerned.

The first months of 1992 saw all the allergen companies engaged in the preparation of the dossiers. In the end, all the documentation consisted, for each company, of 9 boxes of documents for each of the 13 allergen families, making a total of 117 boxes. This documentation was delivered by all the companies to the Ministry within the legal deadlines.

4. The regulatory status of allergens in Europe

It is necessary to make a brief premise about allergology practice in the various European countries. In the Mediterranean countries (Italy, France, Spain, Portugal, Greece), allergologists try to deepen allergological diagnosis as much as possible and consequently adapt immunotherapy to the patient. Therefore, the use of personalised vaccines, often with mixtures of two or more allergens (today much less so, due to the emergence of sublingual therapies) is quite widespread. In Central-Northern European countries (Germany, Belgium, the Netherlands, Denmark, Sweden, Norway, Finland), allergists use standard therapies with a single allergen, or mixtures of allergens from the same family (e.g. grasses, birch-honey). Great Britain deserves a separate analysis. In this country, allergology is at a very low level. Until a few years ago there was no Postgraduate School in Allergology (the first one was established a few years ago in London). In 1986, an event occurred that changed the course of things. The Committee for Safety of Medicines, under pressure from an MP, mercilessly published in the British Medical Journal a list of all the serious reactions from immunotherapy (at that time only injective) that had occurred in the previous years, and recommended very strong restrictions on the use of immunotherapy itself, with consequences for immunological practice in Great Britain that are easy to imagine, quantifiable in a decline in the practice of immunotherapy of over 90%.

We can therefore consider Europe divided into two parts: Mediterranean Europe and Central-Northern Europe.

In France, the use of practising customised vaccines (called '*Allergènes Préparés Spécialement pour un Individu*', APSI) is so entrenched that this is the only form reimbursed by the National Health Service. Consequently, the French Ministry of Health, which has an agency that deals specifically with allergy products, has enacted a law that provides for the registration of intermediate products (called 'bulk') with which manufacturers prepare customised vaccines, APSI (4). This procedure is followed both for vaccines consisting of allergen mixtures and for single-component, i.e. standard vaccines.

In Spain, a Royal Decree similar to the Italian DL No 178 was issued, but this was not followed by the equivalent Decree 13.12.1991 (5). In Spain, as in Italy, ministerial authorisation is issued for the production of allergens, but not for the marketing authorisation of the finished product. In Portugal and Greece there are no manufacturers of allergen products, which are imported.

As far as Central-Northern Europe is concerned, a separate analysis has to be made for Germany, where both non-registered customised immunotherapy vaccines and registered standard vaccines coexist. In contrast, *in vivo* diagnostic extracts (e.g. prick tests) are all registered. These products are not authorised by the Ministry of Health, but by the Paul-Ehrlich-Institut, a prestigious institute based near Frankfurt and specialised in blood derivatives, vaccines and allergens (Fig. 1).

In other Central and Northern European countries, however, only vaccines that are registered, produced and controlled according to the Nordic Guidelines are used (6). Marketing authorisation

(registration) is granted on the basis of dossiers similar to those required for medicinal products (prepared according to Notice to Applicants, today Common Technical Document). The preparation of these dossiers requires a great deal of technical and financial effort on the part of the companies; therefore, the registration procedure is only applied to the most widely used allergy products and therefore high turnover.

Analysing allergen production in Europe as a whole, it emerges that 70-80% of immunotherapy vaccines are produced and marketed in a customised (named-patient) form, while the remaining 20-30% are registered; thus, they are produced in a standard manner similar to pharmaceutical specialities (industrially prepared) and distributed through pharmacies (7).

5. Quality regulations and the Pharmacopoeia

5.1 The Note for Guidance on Allergen Products

In February 1991, a working group on allergen control and standardisation was set up at European level within the Committee for Proprietary Medicinal Products (CPMP), consisting of experts from regulatory bodies, including Dr. Carlo Pini from the *Istituto Superiore della Sanità*. They drafted a Guideline, the Note for Guidance on Allergen Products, which received final approval by the CPMP in March 1996 (8). In the Introduction it is stated that allergen products are divided into two categories: allergens produced industrially (for diagnosis and immunotherapy) and placed on the market in standard packaging, and allergens produced on the basis of an individual prescription for a given patient (named-patient). The Note for Guidance only applies to industrially prepared allergens. The main aspects of allergen quality are discussed in the document.

- **Control of raw materials**
  For all raw materials, the method of collection or cultivation must be accurately described. For pollen, the content of lead, from vehicular pollution, and pesticides must be controlled. Pollution by extraneous pollens must be limited to 1% for pollen mixtures and 0.5% for single pollens. For mycophytes, efforts should be made to avoid the use of mycotoxin-producing species, and when this is unavoidable any mycotoxin content should be checked by mutagenicity tests. For mites, the use of proteins of animal origin in the nutrient substrate should be avoided. Epithelial allergens from animals should be collected from healthy individuals, free of parasitic infestations and who have not recently been treated with pesticides or other drugs.
- **Description of production processes**
  Production must be clearly described using diagrams (flow charts). All steps must be described: grinding and extraction of raw materials, filtration, clarification, dialysis, concentration, fractionation, sterilisation, lyophilisation, etc.
- **Reproducibility between batches (batch to batch consistency)**
  The manufacturer must demonstrate that it is able to produce several batches of the same allergen with a good level of reproducibility. In order to carry out the necessary controls, the use of an in-house standard (in-house reference, IHR) is recommended, which will be compared each time with the batch under examination. In view of the IHR's role as a yardstick, its biological activity must be accurately determined, with SDS-PAGE analysis, IgE-inhibition, etc., in order to determine the biological activity of the allergen.
- **Controls on intermediates and finished products**
  The total allergenic activity of each batch must be determined by means of an IgE-inhibition test. The allergenic activity must be between 50% and 200% of the declared activity. The range 50-200% may seem excessive to the uninitiated; in reality, the response of these immunological systems varies depending on the logarithm of the dose applied, so this range is reasonably limited. In some cases, the allergen is chemically modified in such a way as to alter its biological activity, as in the case of allergoids, and it may therefore be impossible to determine the allergenic activity in the finished product. In these cases, it is permissible to determine the allergenic activity in the intermediate product immediately prior to the chemical modification. This may also be the case for adsorbed (delayed) vaccines, for which, however, proof of adsorption must be provided.
- **Stability**
  The stability of the product and, in the case of delayed vaccines, the adsorption for the shelf-life of the product must be documented. The allergenic activity at the end of the shelf-life must not be less than 30% of the declared activity. Stability can be tested for each taxonomic family (e.g. grasses, betullaceae, composites), and results can be extrapolated from the other family members. In the cases already

described where it is not possible to determine allergenic activity in finished products (e.g. allergoids), it is acceptable for stability to be determined on products at a stage prior to chemical modification.

- **Tolerability (safety) and efficacy tests**
  The principle of extrapolation between allergens belonging to the same taxonomic family is also accepted for these tests.

5.2 The European Pharmacopoeia

The European Pharmacopoeia has introduced a monograph on allergenic products, which is broadly based on the above-mentioned Note for Guidance on Allergen Products (9). Here too there are recommendations on how to collect allergenic raw materials, on the use of in-house standards (here called IHRP, in-house reference preparations), and on analytical tests for analysing antigenic composition and allergenic potency. Here too, the 50-200% tolerance range is affirmed, both for total allergenic potency and for individual allergenic components. As far as the study of toxicity (abnormal toxicity) is concerned, it is recommended only for allergens obtained from mycophytes and by parenteral administration, excluding the prick test. Finally, the requirements for proper labelling are described

- biological potency and/or protein content and/or extraction weight/volume ratio;
- Recommended route of administration and use;
- preservation conditions;
- when present, the name and concentration of the antimicrobial preservative;
- for lyophilised preparations, the name, composition and volume of the liquid to be added for reconstitution, the shelf life after reconstitution;
- when applicable, an indication that the preparation is sterile;
- when applicable, the name of the adsorbent (e.g. aluminium hydroxide or calcium phosphate).

6. The US anomaly

In the USA, the practice of immunotherapy is considerable. It is estimated that between 1.9 and 2.7 million patients received immunotherapy in 2001, predominantly (78%) by the 3400 allergists and to a lesser extent (9%) by the 2600 ENT specialists (10). In the US, the preparation of vaccines is not done by companies, but by allergists themselves in their practices, mixing different quantities of concentrated extracts, called bulk. Bulks are supplied by the companies, and must be registered with the Food and Drug Administration (FDA). A regulatory position diametrically opposed to Europe, where customised (named-patient) vaccines are automatically excluded from the registration procedure, as well as from the scope of the Note for Guidance (see 5.1). Moreover, it is difficult to understand how this allergy practice can be accepted by the health authorities, since the preparation of the vaccines (which are sterile injectable solutions or suspensions) is done in environments lacking the pharmaceutical characteristics that are indispensable for the processing of medicines.

Allergy practice in the US also differs from that in Europe in the large number of different allergens they put in vaccines: 7-8 and even more, whereas Europe is known to limit itself to 2-3 allergens from different families.

7. Conclusions

The quality of allergenic products has improved enormously over the last 20 years, thanks to technological development, thanks to the establishment of dedicated working groups (Committee for Allergen Standardisation, CPMP working group) and thanks to the establishment of periodic meetings between experts from companies and regulatory bodies (*Paul-Ehrlich Seminar*, now in its eleventh edition). At the regulatory level, as we have seen, there are anomalies with respect to medicinal specialities, anomalies due both to the technical peculiarities of these products and to the large number of allergens that need to be made available to allergologists. In fact, the registration process is extremely demanding in operational terms (preparation of the dossier, execution of clinical studies according to GCP) and consequently in financial terms, and it is therefore only possible to resort to it for the allergenic products with the highest consumption and turnover (Gramineae, *Parietaria*, *Dermatophagoides*, Birch, *Ambrosia*, and a few others).

This has created a de facto dual production and distribution channel in Europe: industrially prepared allergy products, usually distributed through pharmacies, and individually prepared products (named-patient, or APSI in France), usually sent directly to the prescribing hospital/doctor or patient. It is important to emphasise that these product categories should not be considered as series A and series B, respectively, in terms of quality. In fact, both production and quality control procedures are the same (11).

At the regulatory level, the two categories follow different paths in some countries, the former being registered

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and the latter unregistered, with the exception of France, which has *ad hoc* legislation for allergen products prepared on an individual basis (APSI). This dual channel is not viewed with much enthusiasm by some regulators. Moreover, there is no doubt that any imposition of registration for all allergy products (as in the Centre-Northern countries, except Germany) would inevitably lead to the disappearance of a large part of allergy lists, with a consequent impoverishment of the quality of professional allergy services.

This particular and politically delicate situation has prompted European companies in the sector to join together in the European Allergen Manufacturers Group (EAMG), whose purpose is not so much to lobby as to publicise the characteristics of allergy products, to promote guidelines, and to talk to the authorities. The hope of many is that a special regulatory regime can be achieved for all those products that, due to their characteristics, cannot be registered according to the normal procedures for medicinal products. It is also hoped that due consideration will be given to the responsible surveillance that allergen companies perform on the correctness of prescriptions and the traceability of the products themselves, surveillance that technically cannot be transferred to the normal distribution channels of medicines, such as pharmacies.

The resolution of these knots may represent the springboard for the hoped-for relaunch of specific immunotherapy, recently also called 'allergy vaccination'. In fact, the efficacy of immunotherapy both on the allergic disease in progress and in preventing the progression of the disease itself (from mono to polysensitisation and from rhinitis to asthma) is now recognised by authoritative WHO guidelines (12, 13) and meta-analyses (14-16), but it is still widely underused in medical practice.

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It has been a good 15 years since this article was written, and upon re-reading it, I was impressed by how much the topics succinctly covered should still be considered valid and relevant today.

Certainly, the work of these intervening years has allowed us to explore specific methodological issues and provide additional elements to improve the conduct of clinical studies on Specific Immunotherapy. To name a few as examples, the definition of seasonality and pollen levels significant for proper exposure, the importance of clinical relevance of effects found, validation of allergen exposure chambers, identification of new biological markers and better profiling of included subjects, appropriate management of "*missing data*", use of electronic technologies to improve data collection, remote monitoring, and prevention of protocol deviations and dropouts. The latest edition of the GCP guidelines (ICH-E6-R3), adapted to the times and increasingly oriented to a "*risk-based*" approach for each stage of study processes, protecting data integrity, safety and dignity of patients, has also moved in the latter direction. Mention should also be made of the entry into force in January 2022 of the New European Regulation for Clinical Trials (No. 536/2014), which provides an innovative, harmonized, transparent and more efficient paradigm at the EU level with regard to administrative aspects and rules to be followed, insisting together with GCP on the qualitative and empowered raising of the elements of conducting clinical research, in the face, however, of major implications in economic terms. Particular focus is on the requirements of Investigators, key players in this scenario, who in order to participate while ensuring high quality standards must necessarily demonstrate adequate training, qualification and experience, adequate facilities for the purpose and strict adherence to protocols, since the outcome of a study is closely tied to these assumptions.

Finally, I had already remarked at the time how important it was to move beyond a view based only on information gained in the context of experimental models, which, while fundamental as "*proof of concept*", do not allow for the assessment of the so-called "*external validity*" of a curative approach. Therefore, move from the concept of "*efficacy*" to that of "*effectiveness*", as also more recently revived in favour of the use of large health databases, registries, independent observational studies and "*pragmatic trials*".

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Clinical studies for SLIT: importance of methodology

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Clinical Trials on SLIT: the importance of the methodology

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SUMMARY

Keywords and acronyms:
• SLIT • sublingual immunotherapy • methodology • clinical trials
• study design, quality of evidence

The aim of this article is to argue and discuss key issues recently raised regarding new approaches for conducting clinical trials of sublingual immunotherapy (SLIT). The clinical efficacy of SLIT in respiratory allergy is supported by numerous randomised, double-blind clinical trials and confirmed by meta-analyses. Nevertheless, limitations in the performance and editorial description (reporting) of some studies may explain the difficulties in assigning an appropriate role to SLIT in the context of guidelines for the treatment of allergic diseases. This issue led the international scientific committees to discuss and reach a consensus whose fundamentals should be respected during the drafting of the study design, its execution and reporting. It was found that particular attention should be paid to aspects of methodology.
In order to ensure the robustness of the results, recommendations were made with regard to patient selection, baseline assessment, statistical analysis, choice of primary and secondary endpoints, and allergen-specific vaccine types. When reporting on clinical trials, it is strongly recommended to follow the CONSORT checklist, together with special measures to reduce the probability of bias. Since safety is a crucial aspect, uniform classifications of adverse events are also desirable to support globally reliable evaluations.
Clinical studies with rigorous methodology and characteristics, extended to all major allergens, are needed to provide clear evidence of the short-term and disease-modifying effects of SLIT. Finally, clinical trials are desired to study the actual efficacy of this treatment in real-life settings.

CLINICAL STUDIES AND META-ANALYSIS: ROOM FOR IMPROVEMENT

Specific immunotherapy is a practice that involves administering allergenic extracts (or vaccines) to allergic patients in order to achieve hyposensitisation towards the allergen itself (1), and thus reduce symptoms in the case of natural exposure. Due to its favourable safety profile, sublingual immunotherapy (SLIT) has attracted the progressive interest of researchers and physicians in recent decades as a valid alternative to traditional subcutaneous immunotherapy (SCIT) for the treatment of respiratory allergy (2). The efficacy of SLIT has been extensively investigated in the past. Over the past decade, due to the large number of randomised clinical trials (RCTs) available, systematic reviews with meta-analyses have been conducted to examine the effects of SLIT on a larger population (3). These studies showed a significant overall benefit compared to placebo. Nevertheless,

some discrepancies between the results and the difficulty in excluding possible publication biases led to some doubts as to their reliability (4). On the other hand, it is not surprising that differences may exist between reviews published in different years, examining different clinical scenarios, including different studies, with different methods for data extrapolation and analysis. Moreover, consistent results were also found, indicating the inadequacy of generalised judgements (5). The interesting fact is that all the meta-analyses showed significant heterogeneity between the included studies, which can be explained by the clinical and methodological variability of the studies (3). However, there is no substantial or formal evidence that the possible sources of clinical heterogeneity can actually affect the course of treatment over a relatively short follow-up period. On the contrary, the methodological limitation of some clinical studies, especially those conducted in the paediatric population and older ones, can be considered a well-founded negative aspect. These observations are confirmed in a recent meta-analysis on SLIT against grass pollen, where the drop-out rate of individual studies, which can be interpreted as a surrogate for methodological quality, significantly affected the overall effect estimate (6). After careful evaluation, however, those studies in the literature, which failed to meet their endpoints, also showed significant limitations (7).

Despite the existence of robust evidence from some large phase III clinical studies conducted on SLIT tablets for grass pollen allergic rhinitis, the above-mentioned reasons explain the attempt of the scientific communities to support, with specific recommendations, the adherence to particular measures to ensure methodological quality in the conduct and reporting of future clinical studies on SLIT (7, 8, 9).

THE METHODOLOGY OF CLINICAL STUDIES ON SLIT

The evaluation and management of allergic disorders is substantially affected by the variability of individual clinical response and exposure to allergens, as well as the subjectivity of symptom assessment. For these reasons, double-blind, placebo-controlled superiority clinical trials should be adopted to study the efficacy of SLIT following the principles of Good Clinical Practice (GCP) standards and guidelines accepted by governmental regulatory institutions (EMA, FDA). However, due to the variable but overall small sample size, these studies are not suitable to provide reliable information on the safety profile. Therefore, confirmatory clinical studies should be based on long-term controlled studies and post-marketing observations. With regard to the planning, conduct and finally the reporting of clinical studies on SLIT (table 1), several specific considerations can be considered. The following is a summary and argumentation of the main issues raised by recent expert roundtables and international discussion boards (9).

METHODOLOGICAL ASPECTS

The rigorous methodology of conducting RCTs must be ensured by measures to minimise the risk of allocation masking bias and unforeseeable centralised randomisation using computer-generated permutation blocks with a specific list within each site in the case of multi-centre studies. A clear description of stratifications, adjustments and the blinded procedure in relation to participants, investigators and analysts must be reported for the entire duration of the study (figure 1).
The inclusion and exclusion criteria must be strictly adhered to and described in detail.
Clinical studies must be conducted according to the principles of *intention-to-treat* (ITT) analysis and all deviations from assignment or withdrawals must be reported in accordance with the CONSORT flow chart (10). Per-protocol analyses are sometimes plausible due to the fact that the duration of immunotherapy studies can be long with inevitably numerous drop-outs.
Attempts to avoid a bias due to drop-outs are crucial in order to preserve the benefits of randomisation. If the drop-out rate exceeds 20% of randomised patients, great care must be taken when analysing the results.
Post-hoc and subgroup analyses, which are only valid if planned in advance,

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have an exploratory value and the results are not suitable for drawing generalised conclusions; rather, they should be confirmed in specially designed trials.

Table 1 Issues to consider carefully when conducting clinical trials

Aspects of the methodology	• Randomisation/masking of the assignment/blind procedure; • Calculation of studio power; • *Intention-to-treat* (ITT) analysis; • Sensitivity analysis and drop-out analysis; • Unbiased selection of outcomes; • Placebo effect; • Clinical relevance of the results.
Basal assessment	• Pollen count, evaluation of indoor levels; • Non-overlapping seasons.
Patient characteristics	• Appropriate diagnosis; • Basal level of symptoms; • Severity of symptoms; • Comorbidity; • Mono and polysensitisation; • No immunotherapy in the previous five years.
Endpoint	• Symptom + drug scores as primary outcomes; • Secondary outcomes; • Exploratory and surrogate outcomes.
Allergen-specific vaccines	• Standardised; • Known efficacy and content of major allergens in mass units; • Description of dosage regimens.
Security	• Uniform coding of adverse events (MedDRA).
Follow-up	• Duration appropriate to the main outcome; • Open procedure for long-term studies.
Report	• In compliance with the *CONSORT* checklist.

Basal assessment

A possible baseline observation period (at least 1 season for pollens) must be arranged in order to include patients who develop a suitable number of symptoms before being randomised and to exclude those without a clear increase in symptoms during the season, or patients with out-of-season symptoms (11).

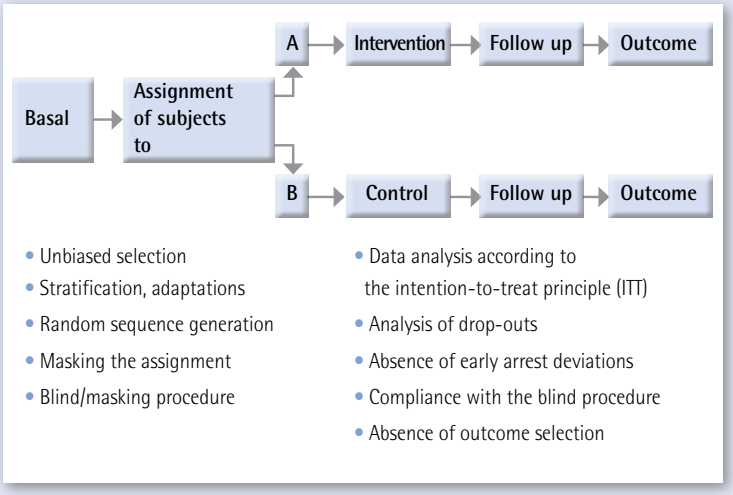
Pollen counts are crucial and the clinical effects of SLIT should be recorded throughout the season. However, the unpredictability and variability of exposure may limit the value of information obtained from a baseline period. Furthermore, the assessment of symptomatology prior to the start of treatment in these patients is not always feasible, as a course of SLIT usually starts at least 8 weeks before the start of the season (12). To ensure compatible and well-defined seasons, studies must involve similar sites. In the case of large-scale multicentre studies conducted in different geographical locations, with high variability of pollen counts and seasons, data should be normalised for the two-week peak period.

In mite allergy, baseline data must be evaluated in conjunction with serially measured fluctuations in indoor allergen levels throughout the duration of the study. Environmental prevention measures are suggested, although their role is a matter of debate. A gradual reduction in mite levels should be planned over the course of the studies.

Patient characteristics

Since it is essential that the causative role of allergens is documented, IgE-mediated sensitisation must be precisely confirmed in patients eligible for studies, either by skin or serologically. The onset and duration of symptoms must be related to sensitisation for at

Figure 1 Characteristics to be observed during the study to ensure an appropriate methodology



least two consecutive years. This clinical relevance may possibly be assessed by specific provocation tests in particularly unclear cases.

The disease must be classified by severity and duration according to the most recent guidelines: ARIA for allergic rhinitis and GINA (*Global Initiative for* Asthma) for asthma (13, 14). Since in real life most allergic patients are polysensitised, a precise differentiation of mono- and polysensitised subjects is essential, also considering cross-reactivities between allergens. Monosensitised patients, or patients who are polysensitised to non-cross-reactive allergens without overlapping seasonalities, are ideal for a single-allergen study. In addition, comorbidities must be carefully examined to avoid misleading results and to more precisely define 'difficult' patients (SCUAD - *severe chronic upper airway diseases*).

Patients must not have undergone immunotherapy in the previous five years.

Endpoint

Since the definition of surrogate markers of clinical efficacy for immunotherapy is still a matter of debate, studies usually adopt the reduction of total symptoms (nasal, ocular and bronchial), individual symptoms and the reduction of rescue medication taken. Symptoms are usually measured on a 4-point scale (from 0 - absent, to 3 - severe), retrospectively or instantaneously, and averages are reported daily, weekly, monthly or seasonally. An alternative approach is the use of a visual analog scale (VAS).

As symptoms and the use of rescue drugs are two closely related endpoints, their association in a single score may be advantageous, but there is currently no standardised method yet. It is important that there is a common approach in assigning different values to different drug categories.

Individual symptom scores, overall patient or physician ratings of response to treatment, symptom-free or medication-free days, objective assessments such as peak nasal inspiratory flow, rhinometry, lung function, change in bronchial specific reactivity (BHR) and blood parameters should be used as secondary outcomes.

Recent recommendations suggest that particular attention should be paid to the assessment of *Patients' Reported Outcomes* (PROs) (15). The effect on quality of life, investigated by means of validated specific and non-specific questionnaires, reflects in fact an impact of the disease that is not detected by organ-specific symptoms or the use of drugs.

For an efficacy claim in asthma, RCTs with a specific design must be conducted. Bronchial symptoms should be used as the primary outcome, in combination with VEMS (forced expiratory volume in 1 second) or PEF (peak expiratory flow). Asthma

control, number of exacerbations, inhaled steroid consumption, quality of life and bronchial reactivity seem to be appropriate secondary outcomes. Furthermore, as uncontrolled severe asthma is considered a contraindication to immunotherapy, strict safety monitoring must be performed.
In some clinical studies, exploratory endpoints may examine progressive changes in the allergen provocation test, changes in skin tests, specific immunoglobulins, immunological parameters, non-specific bronchial reactivity, induced sputum and nitric oxide exhalation.

Power calculation, effect measurements and study duration

Small studies are known to be potentially misleading due to the risk of overestimating the effect size of the intervention or of not noticing small effects (type II statistical error risk). Recent Phase III clinical studies have shown that a number of 150-200 patients per group is adequate for a double-blind randomised controlled trial (16). An appropriate a priori calculation must be made on the basis of the primary outcome to estimate the variability indices and the expected intensity of the effect in order to ensure that a difference from the control is shown. An estimate of the drop-out rate will finally provide the necessary number of patients to be enrolled. With regard to the interpretation of the results, it is not sufficient to use p-values alone to assess statistical significance, but average measures with confidence intervals are required. An effect at least 20 per cent higher than placebo was arbitrarily selected as a reliable cut-off for assessing clinical significance. Furthermore, when baseline data are available, it is suggested to calculate the relative improvement produced by SLIT and placebo in comparison. A valid method is the measurement of the 'area under the curve' for the entire time period. The duration of the study must be adapted to the main outcome. For studies exploring symptomatology, short-term follow-up is sufficient, but to assess long-term, disease-modifying effects, at least 2-3 years must be considered.

Placebo effect

Due to the significant and long-lasting (up to 2 weeks) local side effects of SLIT, compared to the placebo preparation, the appropriate blinded conduct of studies may be compromised. On the other hand, neither histamine nor other substances cause the same allergen effects in the oral mucosa. They are therefore not useful for simulating an active preparation. This issue is currently unresolved and must be considered during efficacy evaluations, as it may produce a bias.

Vaccines

In clinical trials, it is desirable that standardised vaccines with known efficacy and shelf-life be used, labelled with their content of major allergens in mass units (micrograms per millilitre) (17). Vaccines are often labelled in units of biological potency based on skin tests with reference to their own intra-company standard, but the precise measurement of major allergens is a desirable goal. The daily, weekly and cumulative dose, expressed in micrograms, of the major allergens must be carefully reported. Some *dose-finding* studies with grass pollen vaccines have shown a realistic dependence of SLIT efficacy on dose, and it appears that a daily maintenance dose of between 15 and 25 µg of unmodified major allergen is required. With allergoids even lower dosages may be adequate due to their favoured biodistribution. However, there is an urgent need to establish the optimal dose to achieve maximum efficacy, without side effects, for all other relevant allergens as well.
Single allergen vaccines are preferred, but in the case of mixtures, only homologous allergens of proven stability should be used, if combined. Each study must report the induction protocol used, specifying the number of doses per week and the maintenance regime adopted (co-seasonal, pre-seasonal, perennial) with detailed reference periods.

SECURITY: SPEAKING THE SAME LANGUAGE

The evidence of tolerability must come from observations conducted in the pre-marketing phase, but must be continuously confirmed in real life by a post-marketing control system.

Evaluation of the existing literature suggests the need to standardise the way adverse events are classified and reported.

Adverse reactions should be coded using the MedDRA (*Medical Dictionary for Regulatory Activities*), and during the first month of treatment the safety profile should be recorded every day. The MedDRa is currently the only internationally recognised approach for classifying adverse events from phase I studies to monitoring studies (18).

Recently, the importance of a uniform classification and definition of adverse reactions to immunotherapy prompted the European and American task forces, supported by the World Allergy Organisation, to develop a sharing document (19). However, the local implementation phase is set to play a crucial role.

LONG-TERM CLINICAL AND PREVENTIVE STUDIES

SLIT has been shown to have additional effects compared to drug therapy, including long-term efficacy after its discontinuation, prevention of new sensitisation and reduction of the risk of developing asthma in children with allergic rhinitis. Nevertheless, this evidence is currently derived from only a few studies, not entirely free of methodological flaws. Confirmation of these effects must be a priority for future studies with an appropriate design. Since they require prolonged evaluations over time, with the likely risk of high drop-out rates especially in placebo groups, planning extended follow-up phases (3-6 years) is desirable. Since it is unethical to maintain the procedure blinded for many years, the open modality seems to be the best opportunity. The effects of the intervention have to be assessed using the same primary outcome discussed above and measured, even afterwards, in the same reference periods. To examine the appearance of new sensitisation, it is essential to perform the diagnostic tests with the same techniques and extracts.

CLINICAL STUDY REPORTS

In the meta-analyses on SLIT, the possibility of publication bias could not be excluded. This phenomenon stems from the tendency to treat reporting of positive experimental results differently from negative or inconclusive ones. It is strongly recommended that researchers report the results of all studies conducted, regardless of their outcomes. Editorial policies of scientific journals should help to avoid selection bias by discouraging the acceptance of studies not previously registered in specific databases or with regulatory committees (FDA, EMA).

The financing of the study must be clearly indicated. Furthermore, it is important that the results are reported both numerically and with the aid of graphs to improve transparency and post-hoc analyses. The tendency to select favourable outcomes should also be avoided.

The *CONSORT statement* represents the set of minimum recommendations for reporting RCTs. It offers authors a uniform way of reporting results, facilitating their complete and transparent description and supporting their critical analysis and interpretation.

CONCLUSION

The superiority, randomised, placebo-controlled study model provides an estimate of the absolute effect of the therapy, but is not particularly suitable for examining the clinical relevance of this effect. Multiple factors condition the external validity (generalisability) of RCTs: patient characteristics, study condition, treatment regimens, costs, compliance, comorbidities and concomitant treatments. For practical reasons, RCTs always fully consider these factors. For these reasons, the term '*efficacy*' (effectiveness in clinical trials) has been distinguished from the term '*effectiveness in clinical practice*'. Effectiveness profile studies (explanatory studies) determine whether an intervention produces the intended result under ideal circumstances. Observational studies are often used to demonstrate real-world effects. Nevertheless, it is widely documented that these experimental models tend to overestimate results. Efficiency studies in clinical practice (pragmatic studies) represent a valuable tool, as they are characterised by hypotheses and designs formulated on the basis of routine clinical practice conditions and outcomes that are essential for clinical decisions, while retaining those aspects

that guarantee the internal validity of the study (randomisation, masking).

With regard to SLIT, after the conclusive demonstrations of clinical efficacy, the examination of efficiency will be a key point. The latter is indeed profoundly influenced by multiple factors. In particular, lack of treatment compliance may be a major drawback. Since the treatment is mainly taken at home, the risk of interruption by the patient seems realistic. Treatment compliance is much better in RCTs than in real life, hence the need to conduct real-life studies to make this type of assessment, possibly accompanied by pharmaco-economic analyses. Indeed, it has recently been shown that the economic burden of SLIT cycles can affect treatment adherence. On the other hand, the costs of a treatment are increasingly indicated as a relevant aspect to be considered in the balance between desirable and undesirable effects of medical interventions (20). Finally, rigorous analyses from pragmatic studies in real-world settings and safety studies, together with evidence on patient reported outcomes (PROs), will support appropriate and informed recommendations in the context of shared guidelines.

Table 2

Relevant messages
The methodology of many SLIT studies was mostly considered insufficient, until recent pivotal studies conducted on a large scale.
The clinical efficacy of SLIT is well established for grass pollen rhinoconjunctivitis, but more studies are needed for other allergens and asthma. More studies are needed in children.
Rigorous methodology in the design of clinical studies is a priority. Attempts must be made to avoid statistical errors and biases in the conduct, interpretation and reporting of studies.
The items in the *CONSORT* checklist provide important support. The use of the flow chart, which visualises the progress of all study participants, is crucial.
A primary endpoint reflecting both symptom severity and rescue medication is strongly recommended.
Functional measures and surrogate markers cannot replace primary clinical outcomes, but they can provide supporting evidence. Patient-reported outcomes, such as quality of life, are gaining in importance.
The standardisation and characterisation of vaccines, with regard to the exact content of the main allergen, is crucial.
There is a need to closely monitor safety from phase I to post-marketing studies, using a uniform and standardised classification and nomenclature of adverse events.
Adherence to the SLIT cycle can be a crucial issue, worthy of further investigation. Strategies must be implemented to improve compliance.
The evaluation of cost-effectiveness in clinical and pharmaco-economic studies is important in order to place SLIT correctly in the context of guidelines.

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ENG

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With the discovery of the existence of IgE antibodies (by Mr. and Mrs. Ishizaka, in 1967) and their role in sensitization and the triggering of allergic symptoms, allergologic diagnostics, and thus the identification of allergic individuals, became possible thanks to the development of specific *in vitro* tests with serum detection of specific IgE levels (RAST, or ELISA) or *in vivo* on the skin (prick). The aforementioned tests were and still are based on the use of allergenic extracts, and over time there has been a gradual and steady improvement in their standardization by Manufacturers. The extension of new technologies (SDS-PAGE and immunoblotting) to the field of allergology as well made it evident that the allergenic extracts in use consisted of a mosaic of molecules (allergens), and based on the frequency of recognition by sera of allergic subjects some proved to be more clinically relevant (major allergens), others less so (minor allergens). These evidences have set the stage for the development of new diagnostic tools based on the use of single allergens obtained through the application, also in allergology, of recombinant DNA technology. The availability of single allergens in recombinant form obtained from various allergenic sources (pollen, mites, animal epithelia, food...) has revolutionized allergology diagnostics now known as Component Resolved Diagnosis (C.R.D.).

This article on molecular diagnostics dating back to 2013 bears the signature of Dr. Asero, a long-time allergist and a landmark in the allergology world for his countless publications, in some of which I am honored to have contributed. Always a keen observer of new developments in the field of allergology, the author outlines, while emphasizing the importance as the first level of investigation of the classical skin test based on the use of allergenic extracts, the advantages of new diagnostic tools such as the ISAC microarray (other even more sophisticated tools are available today) that were being proposed at the time. In particular, it is based on a miniaturized enzyme immunoassay platform that allows the simultaneous determination of specific IgE against a gradually increasing number of individual allergens over time, mostly produced in recombinant form. The use of molecular diagnostics has made it possible and still makes it possible to untangle the maze of patients with multi-positivity, being able to distinguish between true allergies and cross-reactivities, where one or more allergens may be present in even taxonomically distant species. The article is accompanied by numerous tables describing allergens of various allergenic sources that may be the cause of possible cross-reactions. It is clear that for the allergist this distinction is essential for the setting of an appropriate course of treatment, that is, in the case of food allergy, for the exclusion of certain foods from the patient's diet. Among the interesting aspects that the author wanted to emphasize (which I think also apply today) I recall the invitation not to abuse molecular diagnostics (e.g., in cases of monosensitive subjects highlighted by the prick it might not make sense) and above all that the test should be prescribed by a specialist who has the necessary expertise to interpret the results correctly.

The microarray in allergology diagnosis

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Diagnosis of allergic diseases by ISAC microarray
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SUMMARY

Keywords and acronyms

• Allergens • Cross-reactivity • Specific IgE • Microarrays • Allergy diagnostics

One of the major problems associated with the use of allergenic extracts for in vivo and in vitro diagnosis is the presence of cross-reactivity between phylogenetically conserved allergenic molecules.
Such cross-reactivities can occur between pollens from the same and distinct botanical families, between pollens and foods of plant origin, between perennial inhalants (mycophytes, mites, epithelia), between perennial inhalants and foods, and between apparently unrelated foods. Advances in molecular biology have led to the identification, sequencing, and in vitro synthesis of an increasing number of allergenic proteins.
Currently, the ISAC microarray, an enzyme immunoassay based on modern microchip technology, allows the simultaneous determination of specific IgE for 112 allergens. The aim of this article is to provide some indications for the correct use and interpretation of the results of this new diagnostic tool.

INTRODUCTION

For over a century, allergy diagnosis has been based on the use of allergenic extracts for *in vivo* and *in vitro* testing. These extracts have been progressively and steadily improved over time in terms of purification, sensitivity and standardisation, to the point where it can certainly be said that most of today's allergenic extracts contain the majority of allergenic proteins, at least as far as respiratory allergens are concerned. Nowadays, a positive or negative *in vitro* or *in vivo* skin test with an extract of a respiratory allergen source has a sensitivity and predictive value that is frequently close to 100%. In the field of food allergies, the situation is less bright due to the extreme lability of some allergens and the fact that some allergenic proteins are present in very limited quantities in the respective allergenic sources, with the risk of being present in insufficient concentration in the final diagnostic extract. Despite the aforementioned undeniable merits, allergenic extracts carry with them an ineradicable flaw: they are mixtures of allergenic and non-allergenic proteins and, more importantly, each extract contains, in most cases, more than one allergenic protein. Consequently, the extracts may show some variability in the concentration of individual allergenic proteins from batch to batch and, above all, a positive *in vivo* or *in vitro* test obtained using an allergenic extract does not tell us which proteins present in the allergenic source are responsible for sensitisation. This is certainly a problem when one considers that some allergens are present in homologous form in several distinct allergen sources and are therefore largely cross-reactive.

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Immunological cross-reactivity is associated with the presence of phylogenetically conserved proteins that are widely distributed in the environment and have homologous epitopes. Obviously, if the patient is sensitised to a single allergenic source or to a limited number of allergenic sources, the aforementioned shortcomings of extract-based diagnosis fall by the wayside and do not influence clinical decisions in terms of diagnostic accuracy and subsequent prescription of specific immunotherapy. On the other hand, if the patient is sensitised to numerous allergen sources, e.g. more than 4 distinct pollen species (1), the matter becomes quite complicated, particularly in light of the fact that the pollen calendars of different allergen sources are frequently overlapping. It can be argued that the major problem for the allergist faced with a polysensitised patient is the possibility of co-recognition of cross-reacting allergens present in distinct allergen sources, and that their task is to identify the primary sensitising allergens.

SEASONAL INHALANTS

Among seasonal inhalant allergens (Figure 1), cross-reactivity phenomena are quite complex, occurring at different levels:

a) proteins present within individual pollen species;
b) homologous proteins present in distinct pollen species;
c) so-called panallergens.

Cross-reactivity between proteins within individual pollen species

These cross-reactivities greatly simplify the allergist's task by providing markers of primary sensitisation to a given pollen family. For example, *Phleum pratense* pollen contains all the relevant allergens of the hundreds of grass species, and *Artemisia vulgaris* pollen can be used as a representative of the approximately 13,000 plant species belonging to the Compositae family (2). Similarly, birch pollen serves as a representative of pollen from the Fagales family (including birch, hazel, alder, beech, oak, and hornbeam), pollen from *Cupressus arizonica* represents all of the Cupressaceae (*Cupressus spp.*, as well as *Cryptomeria japonica* and *Thuja spp.*), and olive pollen represents all of the Oleaceae (including ash and privet), and so on.

There are some exceptions to this rule. For example, mugwort and ragweed are part of the Compositae family but have distinct major allergens, which is why they must both be tested diagnostically. Similarly, the Ambrosia group includes different species (*A. artemisiifolia*, *A. trifida*, *A. maritima*), but the major allergens are not completely cross-reacting (3), which is why the pollen to be used diagnostically must be that of the plant that is actually present in a given geographical area.

Cross-reactivity between homologous proteins present in distinct pollen species

With regard to possible cross-reactivities between different botanical species, the best-known example is the cross-reactivity between the major birch pollen allergen (Bet v 1, a group 10 'pathogenesis-related protein') and homologous proteins found in a large number of foods of plant origin; a review of this topic can be found in (4), but there are cross-reactivities in other cases as well. For example, those practising clinical allergology know that in 30% of patients sensitive to grass pollen, skin tests with olive tree pollen are positive even in areas where olive tree pollen is absent; indeed, cross-reactivity has been observed between the group 11 allergens of grass pollen and the major allergen of olive tree pollen (5). Similarly, patients allergic to grass pollen frequently react to *Plantago lanceolata* pollen, whereas sensitisation to the latter in the absence of reactivity to grasses is exceptional; this phenomenon has been attributed to cross-reactivity between allergenic proteins of the

two species (6). The history of the identification of cross-reactivity between ragweed and mugwort pollen is more complex. Both of these plants belong to the botanical family Compositae with distinct major allergens, but the presence of cross-reacting allergens other than pan allergens was identified as early as 15 years ago (7) and, fairly recently, some degree of cross-reactivity between major and minor allergens of both pollens has been observed. Amb a 6 and Art v 3 are homologous (8), as are Amb a 4 and Art v 1 (9), and Amb a 1 and Art v 6 (10).

Interestingly, Amb a 6/Art v 3 co-recognition appears to be unidirectional, since it is only observed in subjects primarily sensitised to ragweed but not in those primarily sensitised to mugwort (8). With regard to Amb a 1/Art v 6 co-recognition, this appears to be bidirectional and is probably the main cause of co-recognition between ragweed and artemisia, at least in northern Italy (Citterio S, et al. In preparation).

Figure 1 Seasonal aeroallergens

Allergen Component	Allergen Source	Latin Name	Protein Group
nCyn d 1	Bermuda grass	*Cynodon dactylon*	Grass group 1
rPhl p 1	Timothy grass	*Phleum pratense*	Grass group 1
rPhl p 2	"	"	Grass group 2
nPhl p 4	"	"	
rPhl p 5	"	"	Grass group 5
rPhl p 6	"	"	
rPhl p 7	"	"	Polcalcin
rPhl p 11	"	"	
rPhl p 12	"	"	Profilin
rAln g 1	Alder	*Alnus glutinosa*	PR-10 protein
rBet v 1	Birch	*Betula verrucosa*	PR-10 protein
rBet v 2	"	"	Profilin
rBet v 4	"	"	Polcalcin
rCor a 1.0101	Hazel pollen	*Corylus avellana*	PR-10 protein
nCry j 1	Japanese cedar	*Cryptomeria japonica*	
nCup a 1	Cypress	*Cupressus arizonica*	
nOle e 1	Olive	*Olea europaea*	
nOle e 7	"	"	Lipid transfer protein (nsLTP)
rOle e 9	"	"	
rPla a 1	Plane tree	*Platanus acerifolia*	
nPla a 2	"	"	
rPla a 3	"	"	Lipid transfer protein (nsLTP)
nAmb a 1	Ragweed	*Ambrosia artemisiifolia*	
nArt v 1	Mugwort	*Artemisia vulgaris*	
nArt v 3	"	"	Lipid transfer protein (nsLTP)
rChe a 1	Goosefoot	*Chenopodium album*	
rMer a 1	Annual mercury	*Mercurialis annua*	Profilin
rPar j 2	Wall pellitory	*Parietaria judaica*	Lipid transfer protein (nsLTP)
rPla l 1	Plantain (English)	*Plantago lanceolata*	
nSal k 1	Saltwort	*Salsola kali*	

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Cross-reactivity between panallergens

a) Polcalcins

Polcalcins are calcium-binding proteins present in virtually all pollen species. Patients sensitised to polcalcins invariably react *in vivo* and *in vitro* with all pollen species. The

Figure 2 Perennial inhalants

Allergen Component	Allergen Source	Latin Name	Protein Group
rAlt a 1	Alternaria	*Alternaria alternata*	
rAlt a 6	"	"	Enolase
rAsp f 1	Aspergillus	*Aspergillus fumigatus*	
rAsp f 3	"	"	
rAsp f 6	"	"	Mn superoxide dismutase
rCla h 8	Cladosporium	*Cladosporium herbarum*	
rBlo t 5	House dust mite	*Blomia tropicalis*	
nDer f 1	"	*Dermatophagoides farinae*	
rDer f 2	"	"	
nDer p 1	"	*Dermatophagoides pteronyssinus*	
rDer p 2	"	"	
rDer p 10	"	"	Tropomyosin
rLep d 2	Storage mite	*Lepidoglyphus destructor*	
rBla g 1	Cockroach	*Blattella germanica*	
rBla g 2	"	"	
rBla g 5	"	"	
nBla g 7	"	"	Tropomyosin
rCan f 1	Dog	*Canis familiaris*	Lipocalin
rCan f 2	"	"	Lipocalin
nCan f 3	"	"	Serum albumin
rCan f 5	"	"	Arginine esterase
rEqu c 1	Horse	*Equus caballus*	Lipocalin
nEqu c 3	"	"	Serum albumin
rFel d 1	Cat	*Felis domesticus*	Uteroglobin
nFel d 2 Cat	"	"	Serum albumin
rFel d 4 Cat	"	"	Lipocalin
nMus m 1	Mouse	*Mus musculus*	Lipocalin

Phleum pratense polcalcin Phl p 7 appears to be the most cross-reactive of the whole group (11). The two polcalcins currently available for *in-vitro* diagnostic purposes (Phl p 7 and Bet v 4, the birch pollen polcalcin) are excellent markers of sensitisation to this group of proteins in pollen polysensitised individuals (12).

b) Profilins

Profilins are structural proteins present in the cytoskeleton of all plant species, including pollen and plant foods. Their high homology results in the sensitised patient being positive for most pollen extracts (13, 14) with the possible exception of parietaria and cypress, which seem to have a lower degree of homology (15, 16). Currently, four profilins are commercially available for *in-vitro* allergy diagnostics by ISAC microarray: Phl p 12 (the profilin from *Phleum*), Bet v 2 (from birch), Mer a 1 (from *Mercurialis annua*), and Hev b 8 (from the natural rubber latex, *Hevea brasiliensis*). The first two are available for *in vitro* singleplex diagnosis by ImmunoCAP. A date palm pollen extract enriched in profilin (50 µg/ml protein) for *in vivo* use is also commercially available, which has shown excellent results in terms of sensitivity and specificity (16).

CROSS-REACTIVITY IN PERENNIAL INHALANTS

Mycophytes

At least two cross-reacting allergenic proteins have been identified in mycophytes: enolase and manganese superoxide dismutase. The glycolytic enzyme enolase is present in many mycetes and shows considerable cross-reactivity between *Cladosporium herbarum, Alternaria spp., Candida albicans, Aspergillus fumigatus, Penicillum citrinum, Fusarium solani* and *Rhodotorula mucilaginosa* (17). Manganese superoxide dismutase has been identified as a major allergen in *Aspergillus fumigatus* and appears to be able to cross-react with homologous enzymes present in various prokaryotes and eukaryotes, including *Saccharomyces cerevisiae*, natural rubber latex and even humans (4). Obviously, patients allergic to mycophytes sensitised to enolase will test positive *in vivo* and *in vitro* with all mycophytes routinely tested.

Fortunately, even in the case of mycophytes we have genuine sensitisation markers such as Alt a 1 for *Alternaria*, Asp f 1 for *Aspergillus*, and Cla h 8 for *Cladosporium* that will allow us to make a correct diagnosis and prescribe the right immunotherapy treatment for that particular patient. We also have an enolase sensitisation marker, Alt a 6 for *Alternaria*, which will allow us to explain the patient's polysensitisation (figure 2).

Figure 3 Hymenoptera allergens, latex, *Anisakis*

Allergen Component	Allergen Source	Latin Name	Protein Group
rApi m 1	Honey bee venom	*Apis mellifera*	Phospholipase A2
nApi m 4	"	"	Melittin
rPol d 5	Paper wasp venom	*Polistes dominulus*	Venom, Antigen 5
rVes v 5	Common wasp venom	*Vespula vulgaris*	Venom, Antigen 5
rAni s 1	Anisakis	*Anisakis simplex*	
rAni s 3	"	"	Tropomyosin
rHev b 1	Latex	*Hevea brasiliensis*	
rHev b 3	"	"	
rHev b 5	"	"	
rHev b 6.01	"	"	
rHev b 8	"	"	Profilin
nMUXF3	Sugar epitope from bromelain		CCD-marker

Mites

Mites contain several cross-reacting allergens. The group 1 allergens, the cysteine proteases Der p 1 and Der f 1 elicit both species-specific and cross-reactive IgE synthesis (with the exclusion of the major allergen of *Blomia tropicalis*, Blo t 1), while the group 2 allergens Der p 2 and Der f 2 cross-react with the group allergen of *Euroglyphus maynei*. The mite tropomyosin (Der p 10 and Der f 10) is a highly phylogenetically conserved invertebrate panallergen that cross-reacts with crustaceans, molluscs, cephalopods, worms (e.g. *Anisakis*), and insects (e.g. cockroach) (figure 2).

Animals

Mammalian serum albumins (Fel d 2 for the cat, Can f 3 for the dog, Equ c 3 for the horse, but also from cattle, pigs, and rodents) are widely cross-reacting (18-20), so much so that patients sensitised to these allergens can test positive *in vivo* and *in vitro* to a number of mammals (21).

Even lipocalins, skin, salivary and urinary allergens present in mammals, despite a reduced level of

Figure 4 Food allergens of animal origin

Allergen Component	Allergen Source	Latin Name	Protein Group
nGal d 1	Egg white	*Gallus domesticus*	Ovomucoid
nGal d 2	"	"	Ovalbumin
nGal d 3	"	"	Conalbumin/ Ovotransferrin
nGal d 5	Egg yolk/chicken meat	"	Livetin/Serum albumin
nBos d 4	Cow's milk	*Bos domesticus*	Alpha-lactalbumin
nBos d 5	Cow's milk	"	Beta-lactoglobulin
nBos d 6	Cow's milk and meat	"	Serum albumin
nBos d 8	Cow's milk	"	Casein
nBos d lactoferrin	Cow's milk	"	Transferrin
rGad c 1	Cod	*Gadus callarias*	Parvalbumin
nPen m 1	Shrimp	*Penaeus monodon*	Tropomyosin
nPen m 2	"	"	Arginine kinase
nPen m 4	"	"	Sarcoplasmic Ca-binding protein

sequence homology, can sometimes cross-react (22) (figure 2).

CROSS-REACTIVITY BETWEEN FOODS

Cross-reactivities obviously also affect food allergens. With regard to plant-derived foods, *in silico* and *in vitro* studies have shown that in the face of a huge number of potential allergens, sensitisation occurs to a very limited number of allergen families within which cross-reactivity phenomena occur (23-26). In foods of plant origin there are basically three highly cross-reactive allergens; profilins (29-32), PR-10 (pathogenesis-related proteins group 10, homologous to the major birch pollen allergen, Bet v 1) (28-30), and LTP (lipid transfer proteins, PR-14) (31). Most of the cross-reactivities occurring within the other families are quite inconstant and to be evaluated on a case-by-case basis. Of note are cross-reactions between natural rubber latex allergens and plant foods.
With regard to foods of animal origin, the main cross-reactivities typically occur between vertebrate fish, due to co-recognition of the major allergen parvalbumin (Gal d 1 in the case of cod), between molluscs, cephalopods and crustaceans due to sensitisation to the aforementioned tropomyosin, between eggs of different bird species and cow's and goat's milk (Figures 4 and 5).

THE ISAC (IMMUNO SOLID PHASE ALLERGEN CHIP) MICROARRAY

The latest version of this diagnostic tool from Thermo-Fisher/Phadia is based on modern microchip technology and consists of a miniaturised enzyme immunoassay platform that enables the simultaneous determination of specific IgE for multiple recombinant (mostly) and natural allergen proteins, including 112 allergens. Further updates and modifications based on the latest discoveries in the field of allergoimmunology are likely to occur over time. The allergens currently available in the diagnostic test, together with the allergen source from which they are derived and their biological significance, are illustrated in figures 1 to 5.

HOW TO USE THE MICROARRAY CORRECTLY

In view of the not inconsiderable costs of a single ISAC determination,

which, depending on territorial situations, may burden the patient's pocket or regional finances, the prescription of such a test must follow an extremely orderly diagnostic procedure. It would be an intolerable waste to require such a test in a subject who on routine *in vivo* investigations is found to be monosensitised to a single allergen source, even when that allergen source includes numerous allergens (as in the case of grasses), since '*tailored immunotherapy*' does not yet exist (and probably never will exist given the costs of registering individual allergens for therapeutic use) and immunotherapy is carried out using extracts in which (hopefully) all allergens are represented. In an atopic subject, the determination of specific IgE against such an impressive number of allergenic molecules can give rise to a harvest of positive results that are difficult to interpret and often of doubtful clinical relevance. It is therefore essential to interpret the results starting from the clinical evaluation of the patient, i.e. knowing what to look for.

In the polysensitised pollen patient, the first two things to do are:

a) Identify primary sensitisers. That is, answering the question: "*To which allergic sources is the patient really sensitised?*" For this purpose, it will be assessed whether the patient's serum reacts towards the so-called 'primary pollen sensitisation markers': Phl p 1 and Phl p 5 for grasses, Art v 1 and

Figure 5 Food allergens of plant origin

Allergen Component	Allergen Source	Latin Name	Protein Group
rAna o 2	Cashew nut	*Anacardium occidentale*	Storage protein, 11S globulin
rBer e 1	Brazil nut	*Bertholletia excelsa*	Storage protein, 2S albumin
rCor a 1.0401	Hazelnut	*Corylus avellana*	PR-10 protein
rCor a 8	"	"	Lipid transfer protein (nsLTP)
nCor a 9	"	"	Storage protein, 11S globulin
nJug r 1	Walnut	*Juglans regia*	Storage protein, 2S albumin
nJug r 2	Walnut	*Juglans regia*	Storage protein, 7S globulin
nJug r 3	Walnut	*Juglans regia*	Lipid transfer protein (nsLTP)
nSes i 1	Sesame seed	*Sesamum indicum*	Storage protein, 2S albumin
rAra h 1	Peanut	*Arachis hypogaea*	Storage protein ,7S globulin
rAra h 2	"	"	Storage protein, Conglutin
rAra h 3	"	"	Storage protein, 11S globulin
nAra h 6	"	"	Storage protein, Conglutin
rAra h 8	"	"	PR-10 protein
rAra h 9	"	"	Lipid transfer protein (nsLTP)
rGly m 4	Soybean	*Glycine max*	PR-10 protein
nGly m 5	"	"	Storage protein, Beta-conglycinin
nGly m 6	"	"	Storage protein, Glycinin
nFag e 2	Buckwheat	*Fagopyrum esculentum*	Storage protein, 2S albumin
rTri a 14	Wheat	*Triticum aestivum*	Lipid transfer protein (nsLTP)
rTri a 19.0101	"	"	Omega-5 gliadin
nTri a aA_TI	"	"	

The figure continues

Figure 5 Plant-based food allergens

Allergen Component	Allergen Source	Common Name Latin Name	Protein Group
nAct d 1	Kiwi	*Actinidia deliciosa*	
nAct d 2	"	"	Thaumatine-like protein
nAct d 5	"	"	
rAct d 8	"	"	PR-10 protein
rApi g 1	Celery	*Apium graveolens*	PR-10 protein
rMal d 1	Apple	*Malus domestica*	PR-10 protein
rPru p 1	Peach	*Prunus persica*	PR-10 protein
rPru p 3	"	"	Lipid transfer protein (nsLTP)

Amb 1 for mugwort and ragweed respectively, Par j 2 for wall pellitory, Bet v 1 for Betulaceae, Cup a 1 for cypress, Ole e 1 for olive, Sal k 1 for *Salsola kali,* Pla a 1 for plane tree;

b) Check for sensitisation to pollen panallergens (polcalcin and profilin);

c) Check for cross-sensitisation to food allergens;

d) Check for primary sensitisation to food allergens and possible cross-reactivity between proteins of the same family present in different foods.

MICROARRAY PROBLEMS AND SHORTCOMINGS

Although the number of allergenic molecules has gradually increased since the first versions of ISAC, the list of available allergens is far from complete. This is particularly true for foodstuffs of plant origin in which new proteins capable of causing sensitisation in genetically predisposed individuals are constantly emerging, but also in the case of food allergens of animal origin such as crustaceans (32). This means that it is not (and probably never will be) possible to dispense with the old and dear skin tests with fresh food (and possibly also with open or blind oral provocation tests), the only investigation capable of bringing patients into contact with all (or almost all) allergenic proteins. The ISAC immunoassay still presents sensitivity problems intrinsic to its nature as a microchip based on the use of minute quantities of allergen. Cases of false negativity are not uncommon. In this respect, singleplex analysis (ImmunoCAP) offers much better sensitivity (provided, of course, that the allergen in question is available). Finally, it is not a quantitative test, unlike ImmunoCAP, so it cannot provide reliable indications for the follow-up of food allergies typical of children

CONCLUSION

The ISAC microarray is not a routine test and cannot be prescribed by general practitioners or specialists without the necessary expertise to interpret it; its use is reserved for the allergist. In the field of respiratory allergies, the test is to be reserved for particularly complex patients due to polysensitisations in whom indications are sought regarding the prescription of the most correct specific immunotherapy. In the field of food allergy, the instrument's greatest utility lies in identifying the allergenic protein to which the patient is sensitised; in light of the chemical and physical characteristics of this allergen, it will be possible to assess the degree of risk associated with ingesting the food containing the relevant allergenic protein. The correct use of the ISAC microarray requires a good knowledge of allergenic molecules and their clinical significance, as well as some familiarity with cross-reactivity phenomena; these skills require the specialist to be constantly updated.

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Content taken from the original article

Provide information, create a profession

Sublingual immunotherapy in real practice today: comparing experiences

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It is with particular pleasure and emotion that I have accepted to publish, in the space of our Notiziario Allergologico dedicated to the Lofarma Academy, the scientific contribution of Dr. Giada Sambugaro, flanked by her father Dr. Renato Sambugaro, concerning their experience in real practice on specific sublingual immunotherapy in adults and children.
Dr. Sambugaro is one of the pioneers of sublingual immunotherapy in Italy, and Giada, a recent specialist, fully represents the spirit that generated the birth of our Academy, i.e. helping young allergologists to grow professionally by working alongside them and trying to transfer the culture of precision allergology to them, where allergological diagnostics and specific immunotherapy are the cornerstones.

Dr. Franco Frati
Director of Lofarma Academy

We have been dealing with sublingual immunotherapy (SLIT) for many years and would like to present our thoughts on the subject.
SLIT is an allergen-specific desensitisation strategy involving the repeated administration of allergens in the form of tablets or sublingual drops. This approach modulates the immune response and induces tolerance, being particularly indicated for patients with allergic rhinitis, allergic conjunctivitis and mild-to-moderate allergic asthma.
In our experience, we have noted that, compared to the subcutaneous route, SLIT offers a safe and effective alternative, with the advantage of home administration and simplicity of use, favouring better therapeutic adherence, especially in paediatric age, where injection therapy is not well accepted. However, SLIT, with the same efficacy as injection therapy, has many other advantages: the good transportability of the product without having to respect the cold chain and the possibility of starting paediatric desensitisation from the age of 5 years, based on the great safety of the therapy itself. By starting desensitisation early, we gradually extinguish the

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persistent mucosal inflammation that, in paediatric age, is at the root of susceptibility to recurrent respiratory infections, especially in the winter months. A 'healthy' mucosa, in our experience, also defends itself better against external infectious agents as well as the allergens that cause allergic symptoms. With the introduction of tablet formulations with modified allergen, a further improvement in safety and compliance has been achieved without reducing the efficacy of SLIT. Pharmacovigilance data confirm that these formulations present infrequent, predominantly local and minor adverse effects, such as oral itching and, in rare cases, swelling of the sublingual mucosa, and no cases of anaphylaxis have ever been described; this is also confirmed in our case reports. Furthermore, looking at the literature on this in a 2021 paper by Moses, Passali and Di Gioacchino, data from the EudraVigilance (European database on suspected adverse drug reactions) on anaphylaxis from SLIT between 2016 and 2019 were analysed. Of the 82 cases reported for dust mite products, 54 were associated with tablets with native allergen without dose escalation phase and 28 with different formulations (oral solutions, drops or unmarketed oral lyophilisates). No cases, however, were related to tablets or drops with modified allergens. I would like to emphasise that normally in the first months of SLIT, mild local adverse effects may occur, but careful monitoring and targeted management reduce their impact and greatly improve compliance.

Clinical studies and real-world data indicate that adherence to a three-year treatment with SLIT tablets is between 60% and 80%, both in adults and paediatric patients. In addition to safety, other factors determining adherence include perceived efficacy, ease of use and appropriate patient education. In our experience in real world, a prerequisite for starting SLIT is to involve the patient or, in the case of paediatric patients, the whole family, by obtaining the parents' willingness to take over the administration of the therapy or the monitoring of their child's regular intake. The parent, therefore, must also be motivated and must discuss the treatment plan, having understood all the logical steps: from the certainty of the diagnosis to the treatment options with illustration of the most up-to-date data on effectiveness. The patient must be informed about the cost, the possible side effects, the controls envisaged in the treatment plan, and must above all have expectations commensurate with the currently available evidence of efficacy. Constant support in the early stages of treatment can help to further improve compliance and ensure optimal long-term therapeutic results. Subsequently, during the course of treatment, in order to monitor the patient's improvement many works suggest the use of symptom/drug score tables that allow us to assess the reduction in symptoms correlated with the reduction in drug use, which translates into a marked improvement in quality of life, the main goal of treatment. In our clinical practice, I would like to emphasise that, if well managed, SLIT has proven to be very effective both in patients with allergic rhinitis, where it offers significant results by reducing symptoms (sneezing, congestion, itching, rhinorrhoea) and the need for symptomatic drugs, and in asthmatic patients where it allows a reduction in the use of bronchodilators and corticosteroids. However, more attention needs to be paid to asthma patients where, before starting treatment, it is essential to carefully assess the level of asthma control and to carefully monitor the patient during therapy in order to avoid flare-ups or unwanted effects. Personally, we believe that sublingual desensitising therapy is an effective option for the management of the allergic individual, especially in paediatric age, where patient compliance is crucial and where the best results can be achieved. The integration of safe and effective immunotherapy with targeted drug therapy allows optimal disease control, a goal that every allergy specialist should pursue.

Giada and Renato Sambugaro

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