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Fennel *(Foeniculum vulgare)*:
a novel food allergen of the Mediterranean Diet

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Abstract

In recent years, the Mediterranean Diet has been attracting substantial interest, because of the benefit conferred on human health, in particular, with its proved capacity of decreasing the cardiovascular risk and the incidence of neurodegenerative diseases. However, while the Mediterranean Diet, in general, is regarded as hypoallergenic, it also includes potentially allergenic foods, most of which are poorly characterized or, simply, unrecognized.
Fennel (*Foeniculum vulgare* var. *dulce*), for example, a member of the *Apiaceae* family and often consumed as a fresh vegetable, is emerging as one of these novel food allergens of the Mediterranean Diet.
This doctoral thesis deals with the occurrence and clinical features of fennel allergy, in a population with a typically Mediterranean Diet from Apulia, Southern Italy, and the immunological characterization of the proteins involved in fennel allergy.
We studied 189 well-characterized food allergy patients. Among them, 57 patients reported symptoms clearly associated with fennel consumption and exhibited positive skin prick tests (SPT) with fennel extract, consistent with the diagnosis of fennel allergy.
These 57 patients were invited to take part into a focused study, aimed at characterizing fennel allergy in detail. The study protocol included: a thorough revision of the clinical history; *in vivo* tests, consisting in SPT, performed with a commercial extract marketed by Lofarma and with an in-house semi-purified fennel extract (with a protein concentration of 3.5 mg/ml), respectively; prick by prick tests carried out with raw and microwaved fennel, respectively; a blood sample collection for *in vitro* studies; serum fennel-specific IgE detection using both the ImmunoCAP Thermo Fisher assay and a RAST-capture assay developed in our laboratory, upon fennel proteins biotinylation.
As regards laboratory work, apart from the semi-purified fennel extract mentioned above, used in the *in vivo* and the *in vitro* investigations and analyzed by SDS-PAGE, we also generated peach and celery extracts, respectively, used in RAST inhibition experiments.
Moreover, the fennel extract was biotinylated and used for the RAST-capture assay and in RAST inhibition experiments, in order to evaluate the possible cross-reactivity between fennel and peach and between fennel and celery (a well-studied food allergen belonging to the *Apiaceae* family, like fennel).
Finally, we carried out Immunoblotting experiments using the fennel allergy patients' sera as the primary antibody and two anti-human IgE secondary antibodies.
The analysis of the 189 food allergy patients revealed that 81 of them (43%) showed cutaneous positivity to fennel, indicating the presence of fennel-specific mast cell-bound IgE. Of these 81 patients, 57 (30% of the whole food allergy population) reported a significant clinical history, consistent with the clinical diagnosis of fennel allergy.
In the 44 patients (out of 57), who participated in the study, the average skin response to fennel was: 26 ± 18 mm^2, with the commercial Lofarma extract and 59 ± 47 mm^2, with the in-house extract ($p<0.001$).
Moreover, the prick by prick tests elicited an average skin response of 42 ± 43 mm^2, with fresh fennel, *vs* 36 ± 32 mm^2, with microwaved fennel, suggesting that the allergenic proteins involved are essentially thermostable ($p=0.562$).
As for circulating fennel-specific IgE, we measured an average value of 2.9 ± 4.8 kU/l, with the commercial ImmunoCAP Thermo Fisher assay, compared to an average value of 3.4 ± 4.5 kU/l, with the in-house RAST-capture assay ($p=0.507$).
RAST inhibition experiments, carried out on sera from selected patients, revealed the absence of cross-reactivity with peach (in contrast to what had been suggested). To the contrary, pre-

incubation of the sera with either the fennel extract or the celery extract caused RAST to become negative, thus, providing evidence of cross-reactivity between fennel and celery, as expected.
Moreover, 18 fennel extract protein bands were detected when the in-house fennel extract was analyzed by SDS-PAGE, under reducing conditions. Immunoblotting analysis was performed in 40 out of the 44 fennel allergy subjects, in order to detect putative fennel allergen bands. These experiments showed that some bands were recognized more frequently, but also that there were rather diverse immunoreactive band profiles, among these patients. Thus, 3 major immunoreactive bands, putatively involved in fennel allergy, were detected. These bands were: a 33 kDa band (detected by sera from 8 patients), a 45 kDa band (detected by 5 patients) and, finally, a 50 kDa band (also detected by 5 patients).
In conclusion, the results showed that *F. vulgare* can be considered a major food allergen, at least in those Countries where the Mediterranean Diet prevails, accounting for a substantial proportion of all food allergy cases (possibly, up to 30%). Moreover, it is also possible to regard fennel allergy as a well-characterized and self-consistent food allergy, as the results of the *in vivo* investigations and the RAST and RAST inhibition experiments pointed out.

Keywords

Food allergy; *Apiaceae* family; skin prick tests; IgE; cross-reactivity; Immunoblotting

1. Introduction

1.1. The Mediterranean Diet benefit on human health

"The Mediterranean Diet is a set of traditional practices, knowledge and skills passed on from generation to generation and providing a sense of belonging and continuity to the concerned communities".

This is the first reason cited by UNESCO for recognizing the Mediterranean Diet as an Intangible Cultural Heritage, in 2010 (http://www.unesco.org/culture/ich/en/RL/00394).

This most authoritative international organization officially ratified what science has been consistently demonstrating during the prevoius decades.

It appears that UNESCO did not consider the Mediterranean Diet just as a mere collection of some selected foods but, rather, ascribed to the eating habits typical of the Mediterranean basin a cultural-promotional role, as stated in the description of this type of diet: *"The Mediterranean Diet encompasses more than just food. It promotes social interaction, since communal meals are the cornerstone of social customs and festive events. It has given rise to a considerable body of knowledge, songs, maxims, tales and legends. The system is rooted in respect for the territory and biodiversity, and ensures the conservation and development of traditional activities and crafts linked to fishing and farming in the Mediterranean communities of which Soria in Spain, Koroni in Greece, Cilento in Italy and Chefchaouen in Morocco are examples".*

The Mediterranean Diet is a complete and balanced nutritional model, combining various ancient culinary patterns and traditions. Usually, it is represented as a pyramid (Fig. 1.), where plant foods constitute the core of the daily intake, whereas foods from animals are more peripheral.

Examples of traditional Mediterranean dietary patterns, with foods from plants at the center of the plate, include the use of couscous, vegetables, and legumes in North Africa; pasta, polenta, rice, or potatoes, along with vegetables and legumes in Southern Europe; rice, along with vegetables and beans in the Eastern Mediterranean regions.

Fresh vegetables, salads, fruits, nuts, olive oil, low fat cheese and yogurt are consumed frequently and garlic, onions, and herbs are used as condiments. Poultry and fish are also

advisable; instead, sweets, eggs and meat should be eaten weekly and in moderation. A moderate intake of wine (one glass for women and two drinks for men per day, preferably during meals) is recommended, since it is an important source of resveratrol, a polyphenolic compound that plays an anti-inflammatory and anti-atherogenic role (http://www.mediterraneandiet.com/). According to the Mediterranean food pyramid, physical activity is very much encouraged, since it promotes well-being and maintenance of a healthy weight (Bonaccio et al., 2012).

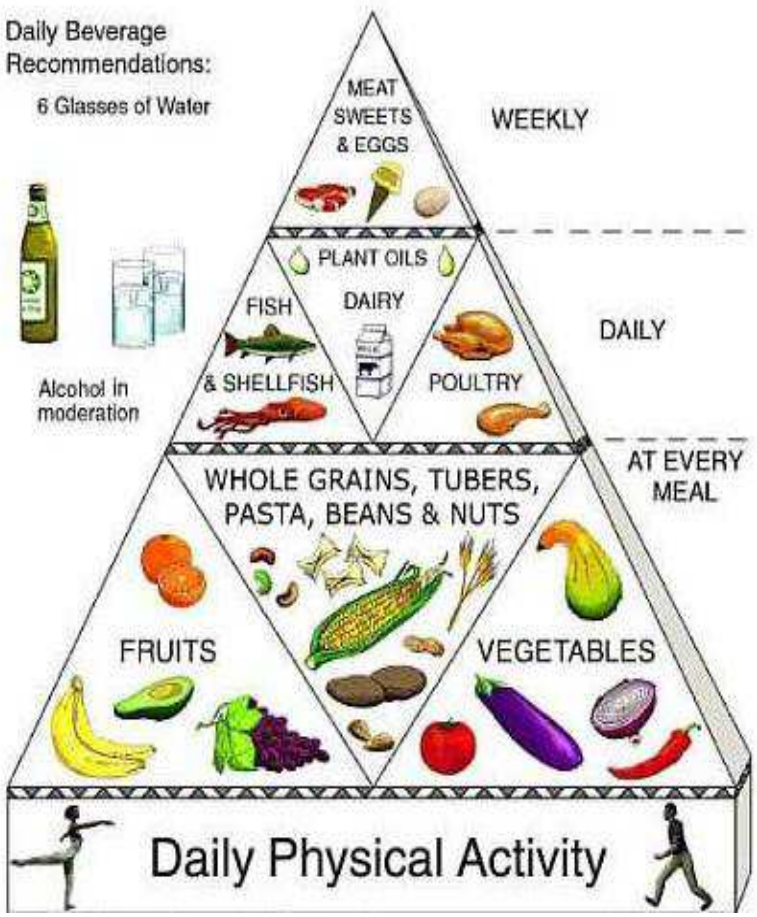

Fig. 1. The Mediterranean Diet food pyramid.

In 2009 an interesting study, published in the "British Medical Journal", underlined that the principal component of the Mediterranean Diet, as a predictor of lower mortality, was the moderate intake of ethanol, followed by low consumption of meat and meat products and high consumption of vegetables, fruits, nuts, olive oil and legumes (Trichopoulou et al., 2009).

Thus, the moderate consumption of alcohol has been considered a key element of the food pyramid, since it has been proven to be involved in the prevention of cardiovascular risk (Costanzo et al., 2010; Di Castelnuovo et al., 2002).

In fact, life-style and eating habits are the major modifiable factors on which it is possible to intervene in order to prevent the onset of the so-called "diseases of wellness" (obesity, diabetes, cardiovascular diseases, tumors), characteristics of our times. Thus, increasing the level of physical activity and introducing healthy food in everyday meals is regarded as highly beneficial for health, therefore affecting positively the quality of human life.

There is a substantial body of evidence linking the Mediterranean Diet to cardiovascular risk reduction and prevention of other major chronic diseases (Willett et al., 2009). A meta-analysis study accounting for a particularly large number of subjects and studies, showed a significant and consistent protection provided by adherence to the Mediterranean Diet, in relation to the onset of major chronic degenerative diseases (Sofi et al., 2010).

The beneficial effects of the Mediterranean Diet have been reported to be due to the synergistic interaction of various constituents. However, numerous studies have demonstrated that single components, such as ω-3 polyunsaturated fatty acids (PUFAs) and polyphenols, play important roles in the prevention of neuro-degenerative diseases and inflammation (Widmer et al., 2015). Inflammation is recognized as the major risk factor in the etiopathogenesis of many chronic diseases, such as cardiovascular diseases (CVD), cancer, type 2 diabetes, Alzheimer's disease and obesity (Ostan et al., 2015).

A recent review, by Barbaresko and co-workers, demonstrated that a diet consistent with the Mediterranean Diet contributes to a reduction of C-reactive protein levels (a marker of inflammation) and leads to an increase of adiponectin (Barbaresko et al., 2013). Furthermore, this diet reduces postprandial inflammation, and the postprandial oxidative stress that can result from postprandial hyperglycemia and hyperlipemia (Camargo et al., 2012).

A link between oxidative stress and inflammation is represented by the transcription factor NF-kB, which is induced by oxidative stress. This factor, in turn, increases the expression of pro-inflammatory genes for cytokines and chemokines (Reuter et al., 2010).

A substantial antioxidant activity is attributed to compounds of plant origin, since these molecules are able to reduce inflammatory responses, as in the case of polyphenols present in extra-virgin olive oil. These polyphenols include hydroxytyrosol, potent antioxidant and free radical scavenger, and oleocanthal, inhibitor of cyclooxygenase activity and best known for its anti-inflammatory properties. Oleocanthal also possesses neuro-protective effects and a

positive effect on markers of inflammation implicated in Alzheimer's disease (Parkinson et al., 2014).

Foods belonging to the Mediterranean Diet are able to prevent the onset of cardiovascular diseases, improving blood lipid profile and reducing blood pressure, presumably through reduced expression of inflammatory biomarkers, reduced oxidation of low-density lipoprotein cholesterol (LDL cholesterol) and neutralization of free radicals (Fig. 2.).

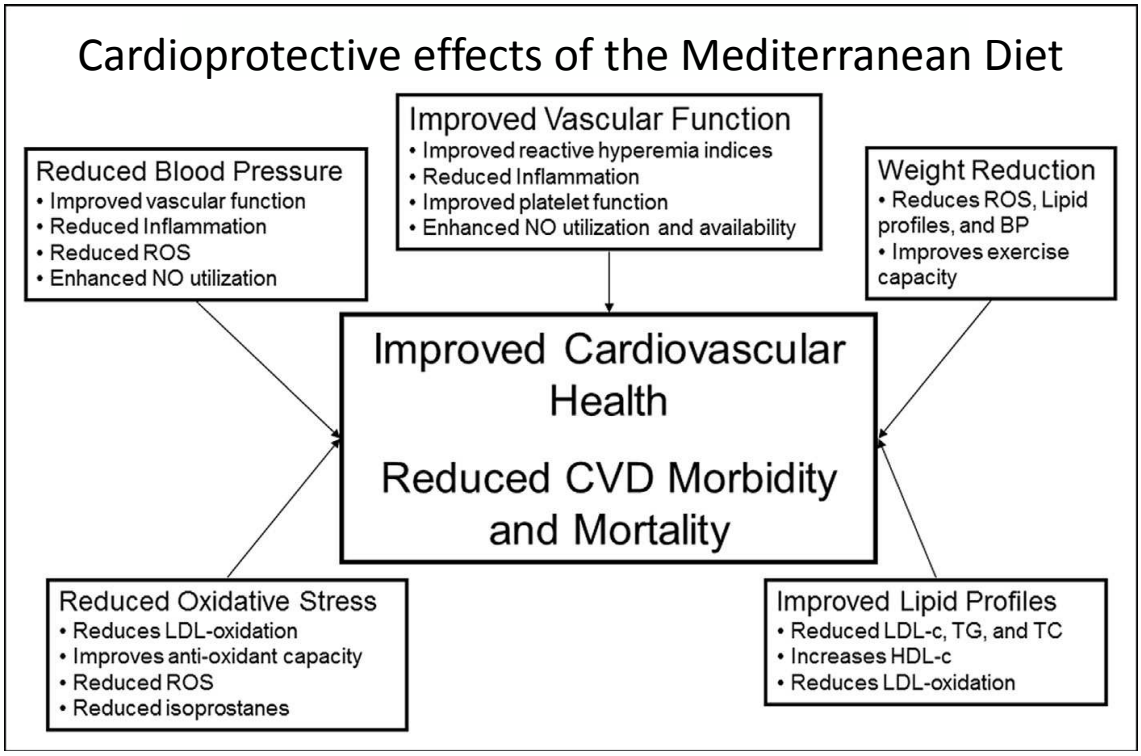

Fig. 2. Mechanisms proposed to underlie the protective effect of foods belonging to the Mediterranean Diet, against cardiovascular diseases.

Moreover, adherence to the Mediterranean Diet showed a protective effect on allergic rhinitis, asthma-like symptoms and atopy (Chatzi et al., 2007). This study was conducted in Crete, Greece, among school children aged between 7 and 18 years. Children in Crete consume high quantities of locally produced fruits and vegetables, whose regular and frequent consumption may have a beneficial role against wheezing and rhinitis symptoms. The results obtained were consistent with results from previous epidemiological studies on the protective effects of citrus fruits, apples and tomatoes on asthma and rhinitis symptoms (Farchi et al., 2003; Garcia et al., 2005; Shaheen et al., 2001). In this study a protective effect of grapes was also

observed. Grapes consumption was shown to be inversely associated with wheezing and allergic rhinitis. Red grape skin contains a mix of non-bioflavonoid and bioflavonoid polyphenols. Oligomeric proanthocyanidins are antioxidants with an anti-inflammatory action that is relevant to oxygen-free radical scavenging, anti-lipid peroxidation and inhibition of the formation of inflammatory cytokines (Li et al., 2001). Moreover, resveratrol, a polyphenolic stilbene found in grapes skin, is able to inhibit cytokine-stimulated inducible nitric oxide synthase expression and nitrite production in human primary airway epithelial cells. It is possible that the beneficial effect of grape intake on wheezing and allergic rhinitis symptoms is mediated by the antioxidant effect of grape phenolic content.

This study also showed a protective effect by nut consumption on the occurrence of wheezing symptoms. Nuts are rich in vitamin E, the body's principal defence against oxidant-induced membrane injury in human tissue, via its role in breaking the lipid peroxidation chain reaction. It was also observed that weekly intake of margarine had a harmful effect on asthma and rhinitis symptoms in this population. Margarine is a source of omega-6 polyunsaturated fatty acids such as linoleic acid, by which arachidonic acid is generated. In turn, arachidonic acid represents the substrate of the cyclooxygenase pathway that leads to prostaglandins bio-synthesis, important pro-inflammatory as well as anti-inflammatory lipid mediators.

Therefore, a general protective effect of a high level of adherence to the Mediterranean Diet on allergic rhinitis, asthma and atopy was assessed. Thus, this study indicated that a high dietary intake of commonly consumed fruits and vegetables and nuts might have a protective effect on the occurrence of asthma-like symptoms and allergic rhinitis.

Because of its benefit on human health, in recent years, the Mediterranean Diet has attracted substantial interest by the biomedical community. In general, foods belonging to the Mediterranean Diet cause allergy less frequently, but the Mediterranean Diet also includes potentially allergenic foods, most of them are poorly characterized or, simply, unrecognized.

Moreover, food allergy, in recent years, has represented the focus of considerable research interest, as epitomized by the inclusion of this topic in the list of the thematic areas of the Sixth and Seventh Framework Programmes of the European Union (http://ec.europa.eu/research/fp6/index_en.cfm; https://ec.europa.eu/research/fp7/pdf/fp7-brochure_it.pdf).

Thus, the Work Programme of Thematic Priority 5: "Food Quality and Safety" of the VI Framework Programme, at point 5.4.2. "Epidemiology of food-related diseases and allergies", read: " The objective is to examine the complex interactions between food intake and metabolism, immune system, genetic background and socio-economic factors to identify key

risk factors and develop common European databases. Many diseases and disorders prevalent in Europe today can be linked to diet, genetic make-up and life-style. Research in this area will use pan-European epidemiological studies concentrating on most important nutrition-related diseases and disorders in order to identify vulnerable population groups, links to diet, genetic factors, and assess how improved diet might reduce the prevalence of these disorders in the future."

Moreover, at point T2.2 – "Epidemiology of food allergy", the same document read: "The focus should be on integrated epidemiological studies on well-defined food allergens influencing the occurrence, prevalence, prevention and distribution of allergic diseases and hypersensitivity disorders in the European population (children, adults, influences during foetal life). Immunological and genetic studies with well-defined criteria for diagnosis, well-defined and/or developed allergy markers and other factors, such as: diet, environment and infections, should be taken into account. The approach should include also an assessment of the socio-economic impact of food allergy, including costs to society and the effect on quality of life of sufferers and their relatives. The generation of new diagnostic tools and methodologies to predict the outcome of allergen interaction with susceptible individuals should be considered. Dissemination plans for patients and the scientific community should be clearly defined".

On the other hand, the Seventh Framework Programme – Knowledge-Based Bio-Economy (KBBE), with the Theme 2 - "Food, Agriculture and Fisheries, and Biotechnologies" Cooperation Work Programme, at point KBBE-2007-2-2-06 – "Impact of exogenous factors in the development of allergy", read: "The aim will be to investigate the mechanisms of early protective effect of exposure to exogenous factors such as diet, life-style and/or living environment on allergy development, using cohort studies and well defined model systems to study the immunological pathways involved, particularly in relation to events in early life. The primary focus will be on the preventable causes, rather than triggers, of allergy onset and on the elucidation of how exogenous factors affect the immune system, mucosal barrier, gut colonization and development of allergy. The expected impact will be represented by the creation of comprehensive and innovative knowledge on the immunological and physiological mechanisms underlying the cause of allergy and its rise in prevalence; promoting health and quality of life of allergy sufferers, by addressing health determinants, such as diet and life-style conditions; finally, providing new knowledge on when exposure to these putative protective agents or mechanisms can improve health by preventing further increase of allergy".

Therefore, within this scientific and cultural framework, particularly important appears the appreciation of the exact magnitude of food allergy. In fact, food allergy is regarded as an increasingly common disorder in Europe and in other developed Countries, but the understanding of the immune mechanisms by which tolerance to food antigens is established or lost is still elusive. Definition of occurrence and socioeconomic impact of food allergy, as well as recognition and characterization of novel food allergens of the Mediterranean Diet, along with elucidation of the immune mechanisms associated with food intake and tolerance, should, therefore, be considered priorities.

1.2. Food allergy pathogenic mechanisms

Food allergy is a widespread problem in Western Countries and, most of the other allergic disorders, seems to be on the rise.

Ingested food represents the greatest foreign antigenic load confronting the human immune system, daily. In the vast majority of individuals, tolerance develops to food antigens. However, when tolerance fails to develop, the immune response may lead to food hypersensitivity, also referred to as allergy, adverse reactions and, sometimes, severe symptoms.

Food hypersensitivity or adverse reactions to food is any abnormal reaction that arises after food ingestion and it can be the result of food intolerance (non-allergic food hypersensitivities) or food allergy. Food intolerances are adverse events due to physiological characteristics of the host, such as metabolic disorders or lactase deficit.

Food allergies, instead, are immunological adverse reactions that may be caused by IgE or non-IgE-mediated immunological mechanisms. However, toxic reactions can mimic food allergy events and typically are due to food intrinsic factors, such as toxic contaminants or pharmacological substances contained in food, which can also cause reactions in healthy individuals, when taken in appropriate doses.

Food allergy consists in various adverse reactions to food, elicited by activation of resident mast cells and circulating basophils, powerful pro-inflammatory cells of the innate immune system, due to interaction of food proteins with food protein-specific IgE, linked to the high affinity FcεRI receptor, expressed on the surface of these cells.

IgE differ from the other four immunoglobulin classes since they possess a supplementary constant domain on their ε heavy chain, called Cε3, which acts as the ligand for a G protein-coupled seven transmembrane domain high affinity receptor (FcεRI), localized on the surface of resident mast cells and circulating basophils.

A mast cell (or a basophil) binds IgE molecules after they are secreted by effector B cells. The soluble IgE antibodies therefore bind to FcεRI receptors on the mast cell surface that specifically recognize the Fc region of these antibodies. The bound IgE molecules serve as cell-surface receptors for antigen. Thus, mast cells (and basophils) possess a set of cell-surface antibodies with a variety of antigen-binding sites. When an antigen molecule binds to these membrane-bound IgE antibodies, it activates a signaling pathway, which ultimately leads the mast cell to release, by exocytosis, the potent pro-inflammatory substances pre-packed in their granules, in particular histamine, as well as newly synthesized mediators (Fig. 3; Alberts et al., 2007).

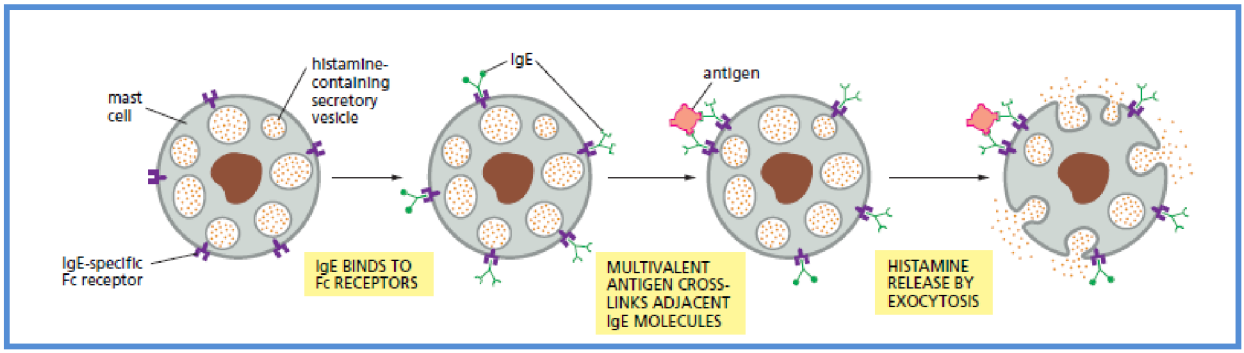

Fig. 3. The role of IgE binding in histamine secretion by mast cells.

Histamine causes blood vessels to dilate and become leaky, which in turn helps white blood cells, antibodies and complement components to enter the site where mast cells have been activated. The release of these mediators by mast cells and basophils is responsible for symptoms of such allergic conditions as hay fever, asthma and hives. Allergic symptoms can lead to clinical manifestations of variable degree of severity, up to anaphylaxis, which represents the most severe outcome of all food-mediated allergic reactions.

Thus, individuals with a genetic predisposition, referred to as atopy, may mount anomalous immune responses to harmless environmental antigens, such as food proteins, leading to

production of substantial amounts of specific IgE antibodies. A protein eliciting an anomalous IgE immune response is called an allergen.

1.3. Food allergy diagnosis

Food allergy diagnosis begins with the collection of patient's clinical history. Based on the information derived from this initial step, diagnosis can be completed and achieved by different laboratory studies.

The importance of clinical history depends on the patient's ability of remembering the symptoms and on the examiner's capacity of distinguishing reactions due to food from those with other etiologies. A tool used in addition to clinical history is represented by diet diaries, where patients report all foods ingested during a specific period of time, in a chronological way and the symptoms. Finally, these diaries are reviewed to determine the relationship between food ingested and symptoms experienced by the patient.

Symptoms associated with allergic reactions can affect the gastrointestinal tract, the skin and the respiratory tract. Gastrointestinal symptoms can manifest with mouth itching, lips and palate edema, nausea, vomiting and diarrhea. The skin symptoms are characterized by generalized itching, erythematous rash, urticaria and angioedema. Respiratory symptoms, instead, can affect the larynx and the upper and/or lower respiratory tract, with symptoms similar to those of allergic asthma and allergic rhinoconjunctivitis, such as periocular itching, conjunctival erythema and lachrymation, nasal itching, rhinorrhoea, laryngeal edema, coughing, wheezing and dyspnea.

Another method also used to collect additional clinical information is represented by the elimination diets, which are employed not only in diagnosis but also in food allergy management, when certain foods are suspected to cause allergic reactions. Thus, these foods can be completely eliminated from the diet. The success of these diets depends on the food allergens identification and on patient's ability to follow a diet free from all forms of the allergen suspected of causing the allergic reaction.

As regards the diagnostic evaluation methods, skin prick test (SPT) are the major tool in detecting patients with IgE-mediated food reaction. SPT are performed by pricking the patient's skin (usually, at the volar surface of the foreorms) through a droplet of allergen

solution, which allows the allergen molecules to interact with potential IgE specific for that given allergen, on the surface of skin-resident mast cells.

The skin usually is punctured using a sterile lancet and the results are read 20' after pricking. Histamine hydrochloride 10 mg/ml in 50% glycerol solution is used as the positive control in skin prick testing, while saline with 1% albumin serves as the negative control. Positive responses consist in a wheal-and-flare reaction, whose magnitude essentially reflects the amount of endogenous histamine released by activated cutaneous mast cells (Fig. 4). To measure the elicited cutaneous reactions, the wheal can be outlined with a felt-tip pen and the outline can be blotted on removable tape and recorded on paper, for convenience. The area can be calculated by superimposing a transparent cellulose acetate sheet with a 1 mm square lattice grid and counting the squares comprised within the outline (each square 1 mm^2; Corallino et al., 2007).

Criteria for food allergy SPT interpretation were determined by Bock and May (Bock et al., 1978). According to these Authors a wheal (elicited by an allergen) with a diameter at least 3 mm larger than that of the negative control, is considered positive. However, positive SPT indicate an immunological sensitization that not always associates with clinical manifestations. Therefore this diagnostic approach is highly sensitive but not high specific (Sicherer et al., 2010). Thus, the positive predictive value of this method is slightly less than 50%. Conversely, negative SPT support the hypotesis of absence of IgE-mediated reactions. Hence, the negative predictive value of this test is greater than 95%, provided that the quality of food extracts is optimal (Bock et al., 1978).

When diagnosing IgE-mediated sensitizations to fruits or vegetables, SPT can be replaced by prick by prick tests, in order to avoid false negatives, since commercial food extracts could be altered by commercial preparation, because of proteins lability. These tests are performed pricking the skin through a slice of the suspected food.

Finally, in routine diagnosis of allergic diseases, there is a second level exam, known as RAST (Radio Allergo Sorbent Test), which allows determination of specific IgE in serum and other biological fluids. This method is characterizaed by a lower negative predictive value compared to SPT, but it has a higher positive predictive value (Asero et al., 2007).

1.4. *Foeniculum vulgare*

1.4.1. Foeniculum vulgare and food allergy

In order to assess the potentially hypoallergenic properties of the Mediterranean Diet, definition of occurrence and socioeconomic impact of food allergy in our area, recognition and characterization of novel food allergens, elucidation of the immune mechanisms associated with their intake and tolerance need to be clarified. These aspects of the problem, of course, in turn, need to be supported by larger epidemiological studies, providing the rationale for the investigative work to develop. Moreover, since increasingly strict regulations on marketed pre-packed foods within the European Union also include allergen specification and labeling, we think that the thorough characterization of novel food allergens of the Mediterranean Diet cannot but add to the general appreciation by consumers of the Mediterranean Diet itself, with the exact identification of the related (possibly marginal) health risks.

In this research project, the attention has been focused on the characterization of a novel food allergen belonging to the Mediterranean Diet: *Foeniculum vulgare*.

Foeniculum vulgare allergy has been studied only marginally, so far. Therefore, fennel is not included in the allergen list of the International Union of Immunological Societies (IUIS) database (http://www.allergen.org/).

1.4.2. Foeniculum vulgare botanical and agronomical characteristics

Foeniculum vulgare, commonly called fennel, is a member of the *Apiaceae* family (also called *Umbrelliferae*), a large group of plants encompassing approximately 300 genera and more than 3000 species.

Fennel is an ancient seasonal herb that grows wild in most of temperate Europe, but is generally considered indigenous to the Mediterranean Countries.

Fennel was well-known to the ancient Egyptians, Greeks and Romans, who grew it for its aromatic seeds and the edible fleshy shoots. In various Italian regions, especially in Southern Italy, in stony and sub-mountainous areals up to a height of 700 metres, and along the coasts, wild *Foeniculum* species with perennial plants, characterized by strong roots that do not form the false bulb (the so-called *"grumolo"*, in Italian) and resistance to drought are still present.

From the alimentary point of view, the young leaves of these wild species are used as flavoring in several food preparations.

The genus *Foeniculum* was described by Miller in 1768, who separated it from *Anethum*, as previously indicated by Linnaeus. Moreover, Miller distinguished two species in the genus *Foeniculum*: *F. vulgare*, which includes *F. vulgare* (var. *dulce*), cultivated as a vegetable for consumption of the *"grumoli"*, and *F. sativum*, cultivated for its seeds. A further subdivision (within *F. vulgare*) was proposed by Tutin in 1968: *F. vulgare* subspecies *piperitum*, whose inflorescences and the stem upper part, are used for pickled storage, and *F. vulgare* subspecies *capillaceum*, whose seeds are employed in alcohol preparations as flavoring.

F. vulgare (var. *dulce*) is a herbaceous plant with a biennial cycle. In the first year, it develops the vegetative part represented by the so-called *"grumolo"* or false bulb which is the edible part, made of the basal sheaths of the leaves, overlapping with each other with the function of accumulating nutrients (Fig. 4.), and by the root system. Instead, during the second year, fennel produces flowers, fruits and seeds (Siviero et al., 2005).

Fig. 4. Fennel *"grumolo"* or false bulb growth during the first year of vegetative cycle.

Fennel is a crop characterized by an autumn-winter production cycle and, therefore, it has reduced water needs. Its ideal minimal temperature is around 7 °C, while the optimum

ranging is between 15 °C and 20 °C. Fennel is also frost-resistant, providing that the temperature does not fall below -2 °C.

Germination requires at least 10 °C, but it is hindered over 30 °C, with an optimum temperature between 20 °C and 25 °C.

The irrigation is an important practice expecially during the early cycle stages, since the plant has to grow in a warm period. Thus, it is particularly sensitive to water stress that can be responsible for physiological problems, besides bacterial/fungal infections.

The most important features for variety choice are the cultivation period, the production area and the cycle duration. Thus, fennel plant prefers deep grounds with an optimal pH between 6 and 7.5.

(http://agronotizie.imagelinenetwork.com/vivaismo-e-sementi/2015/05/04/il-finocchio-una-coltura-per-tutte-le-stagioni/9292).

1.4.3. Foeniculum vulgare nutritional aspects and health properties

Fennel is one of the most pleasing and sweetest vegatebles. It can be consumed either as raw fresh food, in salads, or cooked.

From a nutritional point of view, *F. vulgare* is characterized by a low-energy content, with only 9 kcal/100 g (38 kJ/ 100 g). It is also worth noticing that it is characterized by a high amount of fiber and mineral salts, in particular potassium (Food Data Bank INRAN, Rome). This vegetable also has a high content of vitamin C, equal to 12 mg/100 g, corresponding to 20% of the recommended daily intake (*Recommended Daily Allowance*, RDA). See Tab. 1.

(http://nut.entecra.it/646/tabelle_di_composizione_degli_alimenti.html?idalimento=005320&quant=100).

Nutrient	Amount/100 g
Water	93.2 g
Protides	1.2 g
Carbohydrates	2 g
Fiber	2.2 g
Potassium	394 mg
Calcium	45 mg
Phosphorus	39 mg
Magnesium	16 mg
Sodium	4 mg
Zinc	0.9 mg
Iron	0.4 mg
Selenium	0.9 µg
Vitamin A	2 µg
Vitamin B1	0.02 mg
Vitamin B2	0.04 mg
Vitamin C	12 mg

Tab. 1. Nutritional features and content of vitamins and minerals per 100 g of fennel (edible parts).

Furthermore, phytochemical research carried out on *F. vulgare* has led to the isolation of fatty acids, phenolic components, hydrocarbons, volatile components, and few other classes of secondary metabolites from its different parts. Mostly these phytochemicals are found in essential oil. For example, the anise odor of *F. vulgare* is due to its essential oil content. It makes an excellent flavoring agent in various types of food and food related products. The essential oil of fennel has been reported to contain more than 87 volatile compounds. The accumulation of these volatile compounds inside the plant is variable, appearing practically in any of its parts: roots, stem, shoots, flowers, and fruits. The main components are the trans anethol phenolic ether and fenchone. Other important components, present in various amounts are estragole, p-anisaldehyde and terpenes. All these molecules give the plant not only an intense characteristic flavor, but also interesting pharmacological properties, such as antimicrobial, antiviral, anti-inflammatory, antimutagenic, antipyretic, antispasmodic, antithrombotic, apoptotic, cardiovascular, chemomodulatory, antitumor, hepatoprotective, hypoglycemic, hypolipidemic and memory enhancing property.

In fact, *F. vulgare* is well-known for the cure of numerous infectious disorders of bacterial, fungal, viral and mycobacterial origin.

Finally, not only fennel exhibits pharmacological activities but also reveals some environmental active properties. These activities play a key role in the management of nematode, insect, mosquitoes and some harmful larvae of malaria producing vector. Thus, *F. vulgare* extracts and isolated biologically active compounds are evaluated for their insecticidal, repellent, acaricidal, larvicidal and nematicidal activity (Badgujar et al., 2014).

1.4.4. Foeniculum vulgare production in Italy

Fennel is cultivated exclusively in open fields. According to 2008-2012 ISTAT data, in 2012 *F. vulgare* cultivation occupied 20,000 hectares and 4,898 thousands of quintals of fennel were produced (Tab. 2.).

Cultivation	2012	
	Cultivated area	*Production*
Cereals	3,258	170,235
Tuber plants	58	14,863
Legumes	70	1,387
Tomatoes	92	51,320
Cultivated mushrooms	no data	10,169
Salad	38	7,557
Fennel	20	4,898
Carrots	11	4,823
Cauliflower	17	4,141
Artichoke	36	3,649
Cucumber	9	3,473
Onions	11	3,443
Zucchini	13	3,172
Cabbage	15	3,118
Aubergine	8	2,177

Tab. 2. Last available (2012) ISTAT data regarding some important cultivations in Italy. Cultivated area and production are expressed in thousands of hectares and in thousands of quintals, respectively.

The Italian regions that are most involved in *F. vulgare* production are: Apulia (30%), Lazio (11%), Sicily and Marche (9%), Abruzzo (5%), Calabria (4.5%) and Emilia-Romagna (4%). Italy contributes to about 85% of the so-called *"grumoli"* production, worldwide. In particular, fennel is exported to France, Germany and Switzerland.

Other foreign Countries where fennel grows are Turkey, Syria, Egypt, Morocco and Iran.
It is worth noticing that, although fennel has an autumn-winter production cycle, thanks to the introduction of new hybrids during the last few decades, fennel now is cultivated throughout the year. In particular, *F. vulgare* is characterized by a spring-autumn cycle in the Fucino area (Abruzzo), while it has an autumn-spring cycle in Southern Italy (Campania, Apulia, Molise, Basilicata and Calabria), with different cycles according to the variety. Thus, early varieties have a cycle of 70-80 days; medium varieties a cycle of 90-120 days; medium-late varieties a cycle of 130-160 days and, finally, late varieties are characterized by a cycle of 180-200 days.

1.5. *Foeniculum vulgare* allergy. Background

Fennel allergy has been seldom reported in literature only occasionally and the few publications that have addressed this type of allergy focused on the relationship between fennel allergy and birch and mugwort pollen allergy, in the so-called birch-weed or fruit-spice syndrome (Asero, 1997; 2000). One of these few previous papers regarding fennel allergy, by Jensen-Jarolim E. and co-workers, studied 15 patients with allergic reactions to spiced foods, including foods containing fennel (*F. sativum*) seeds as an ingredient (Jensen-Jarolim et al., 1997). The Authors concluded that Bet v1 and profiling-related allergens could be responsible for allergy to a variety of *Apiaceae* spices, including fennel, thus demonstrating the immunological basis of the clinical association between fennel seeds allergy and birch or mugwort pollen allergy.
More recently, Pastorello and co-workers studied the association between fennel and peach allergy (Pastorello et al., 2013). Thus, since a high number of individuals with fennel allergy symptoms had been observed in a group of severe peach-allergic patients, this study aimed at investigating the clinical and immunological relationship between peach and fennel allergy and, therefore, at detecting and characterizing the putative allergens responsible for this relationship.
In this paper, at first, an in-house fennel extract was analyzed by Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which revealed numerous components with a molecular weight ranging approximately 9 to 100 kDa (Fig. 5.).

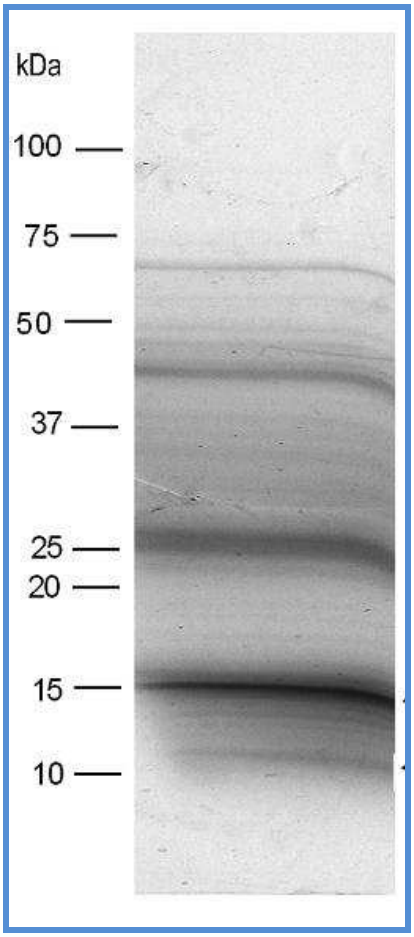

Fig. 5. SDS-PAGE of fennel extract and protein separation according to their molecular weight. The proteins were separated in a discontinuous gel with a 6% stacking gel and a 7.5-20% separation gel, at 6 mA for 16 h (Pastorello et al., 2013).

As it is shown in Fig. 6., sera from 25 fennel allergy patients were used for IgE Immunoblotting analysis and IgE-binding proteins were detected by incubation with an ^{125}I-labeled anti-human IgE antibody. Sixty per cent (60%) of patients' sera reacted toward an approximately 9 kDa band, whereas 44% of the sera recognized a protein of approximately 15 kDa and 96% reacted with bands in the range of 65-75 kDa.

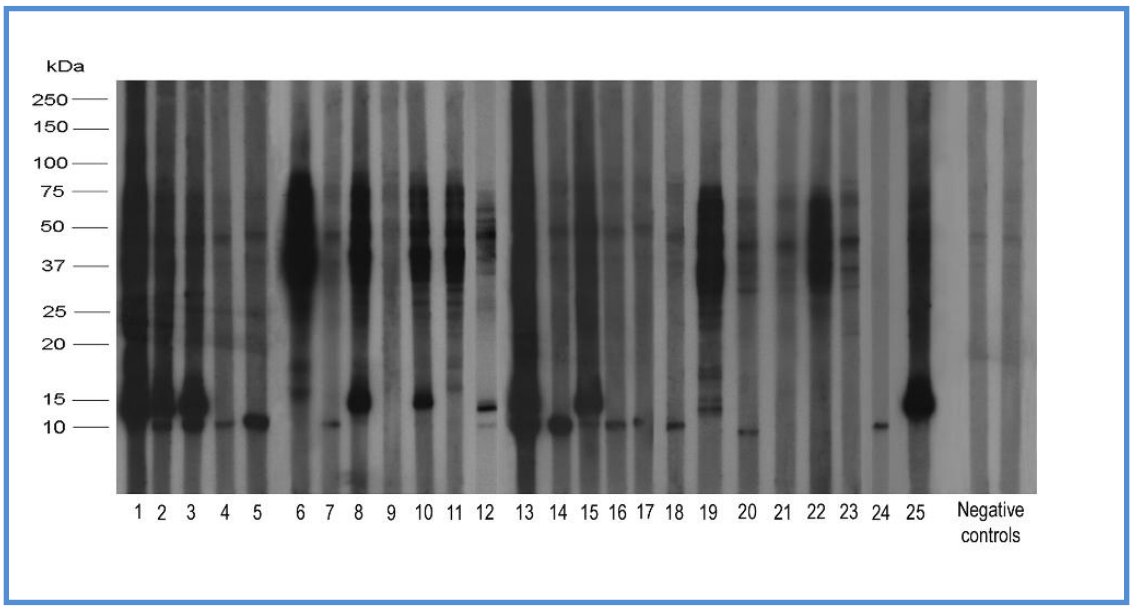

Fig. 6. IgE Immunoblotting of fennel extract using sera from 25 fennel allergy patients and 2 negative controls. After electrophoresis, fennel proteins were electrotransferred to a nitrocellulose membrane, at 0.45 A and 100 V for 4 h at 4 °C. Moreover, sera from the 25 fennel allergy patients and 2 negative controls, diluted 1:5, were used as primary antibody; while IgE-binding proteins were detected by incubation with an ^{125}I-labeled anti-human IgE antibody, diluted 1:2. Exposure to autoradiographic films, at – 70 °C for 4-7 days, was also performed (Pastorello et al., 2013).

An immunoblotting inhibition experiment was also performed to evaluate the cross-reactivity between fennel and peach extracts, using a pool of sera from patients selected on the basis of their Immunoblotting response pattern to fennel proteins. This experiment revealed that pre-incubation of the pool with a peach extract at different dilutions completely inhibited IgE binding to fennel proteins, suggesting a high cross-reactivity between these two plant foods (Fig. 7.).

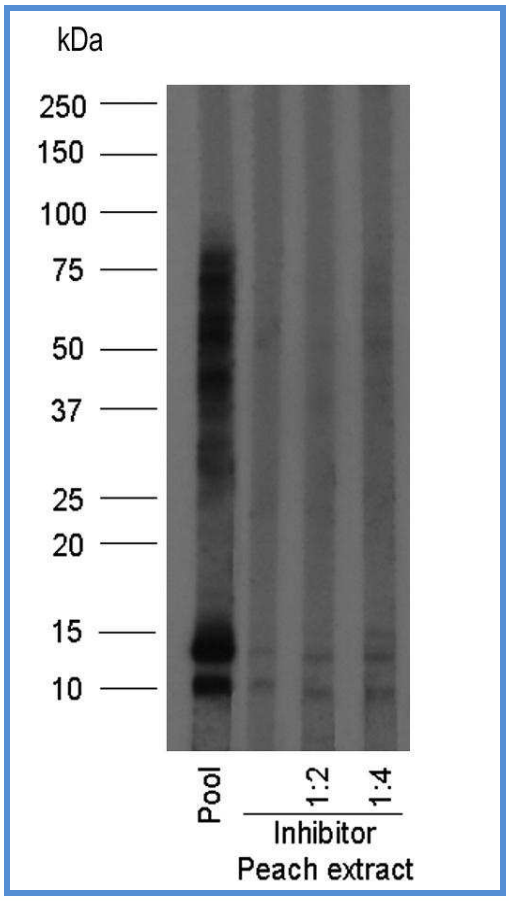

Fig. 7. IgE Immunoblotting inhibition of the ability of binding fennel proteins by pooled sera from some fennel allergy patients, pre-incubated with different concentrations of peach extract (Pastorello et al., 2013).

Moreover, the paper demonstrated that the 9 kDa band could be identified, by Liquid Chromatography - tandem Mass Spectrometry (LC-MS/MS), as a lipid-transfer protein (LTP), characterized by a high homology with Pru p3 protein in peach, suggesting its importance in the cross-reactivity evaluation. For this reason, fennel allergy is considered a LTP-related food hypersensitivity and therefore fennel has been included in the list of foods that cause "LTP syndrome" (Pastorello and Robino, 2004).

1.6. The *Foeniculum vulgare* allergy project

This research project aimed at estimating the occurrence of fennel allergy, in a population with a typically Mediterranean Diet, from Apulia Southern Italy, and characterizing, from an immunological point of view, *F. vulgare* proteins involved in food allergy to this vegetable. To this purpose, a number of patients with well-diagnosed fennel allergy was detected and non-allergic subjects were recruited as negative controls. Fennel allergy diagnosis was based essentially on clinical history and demonstration of IgE toward fennel, achieved *in vivo* by skin prick testing. In particular, quantitative SPT were carried out with a commercial extract and with a semi-purified fennel extract produced in our laboratory, respectively. Also quantitative prick by prick tests with raw and microwaved fennel, respectively, were performed. Both these types of skin tests allow detection of mast cell-bound specific IgE for fennel.

The detection of the lesser fraction of circulating fennel-specific IgE was performed *in vitro*, using the commercial available ImmunoCAP RAST, marketed by Thermo Fisher, and a RAST-capture assay, developed in our laboratory. To this purpose, the in-house fennel extract was first biotinylated (by conjugation with biotin disulphide N-hydroxysuccinimide ester). The biotinylated reagent was used for this ELISA technique. This approach was also adopted in RAST inhibition experiments, in order to evaluate the possible cross-reactivity between fennel and peach and between fennel and celery, respectively.

Finally, the protein profile of the fennel extract was examined by Immunoblotting analysis in order to identify fennel proteins bands that react with IgE from *F. vulgare* allergy patients. Thus, in these experiments, patients' sera and anti-human IgE peroxidase-conjugated antibody were used as primary and secondary antibodies, respectively.

2. Materials and methods

2.1. Fennel allergy diagnosis work up

The diagnosis of fennel allergy was based essentially on: a convincing clinical history; *in vivo* tests (SPT and prick by prick) and *in vitro* tests (RAST).

2.1.1. Convincing clinical history

A convincing clinical history is definitely important in the diagnosis of food allergy.

There are many specific factors to be considered that make a clinical history convincing, in the field of allergology. First, the timing. The allergic reaction onset, after consumption of food regarded as responsible for the allergic reaction reported by the patient, has to be immediate. Thus, since food allergy is based on an IgE-mediated reaction, in most cases, the allergic reaction takes place within few minutes, after the suspected food consumption.

Secondly, the nature of symptoms. The symptoms associated with food allergy reactions can affect primarily the skin (itching, cutaneous rash, urticaria, angioedema). The gastrointestinal tract may be also involved (lips, mouth, tongue and palate itching and edema, nausea, vomiting, diarrhea). Finally, symptoms related to the larynx and the upper and/or lower respiratory tract, may also occur.

Reproducibility. It is also important noticing if the allergic reaction is repetitive, *i.e.*, if it occurs whenever the suspected food is consumed.

Nature of the offending allergen. Moreover, we need to take into consideration the type of food involved in the allergic reaction reported by the patient. Chocolate, for example, very seldom causes allergy (inspite of the general belief at the lay public level). Also important causing is the amount of the suspected food assumed.

Food allergy reactions often occur when having special meals, associated with ceremonies, or when dining out. In both cases, we can eat much more than usual and, sometimes, we do not know if potentially allergic foods are used as ingredients. In addition, alcohol intake as well as physical activity (e.g., dancing, etc.) may facilitate the onset of symptoms associated with food allergy reactions, such as urticarial and cutaneous rash, because of the alcohol ability to determine vasodilation and other similar mechanisms.

These principles were adopted in collecting the anamnesis of the study patients. See also the Results section.

2.1.2. In vivo tests

The *in vivo* tests included SPT and prick by prick. SPT were performed by pricking the patient's skin through a droplet of allergen solution, which allowed the allergen to interact with potential IgE specific for that given allergen, on the surface of skin-resident mast cells. In food allergy diagnosis, usually, a collection of 34 commercial food allergen extracts was used (Tab. 3.). In particular, SPT were performed with commercial extracts marketed by Lofarma (Milan, Italy). An in-house semi-purified 100,000 x g supernatant fennel extract was also used.

In the highly standardized technique of skin prick testing, the skin is usually punctured using an Østerballe and Weeke-type 1 mm point length standardized needle (Østerballe & Weeke, 1979), supplied by Stallergenes. Histamine hydrochloride 10 mg/ml in 50% glycerol solution (from Stallergenes, Antony, France) was used as the positive control in skin prick testing. Saline with 1% albumin served as the negative control. Results were read 20' after pricking.

Positive responses consist in a wheal-and-flare reaction, whose magnitude essentially reflects the amount of endogenous histamine released by activated cutaneous mast cells. In turn, the magnitude of mast cell activation essentially reflects the amount of the existing specific IgE bound to their surface by high affinity receptors (IgE cross-linking, by allergen molecules penetrated into the skin, triggers mast cell activation and histamine release). In quantitative skin testing, to measure the elicited cutaneous reactions, the wheal can be outlined with a felt-tip pen and the outline can be blotted on removable tape and recorded on paper, for convenience. The area is calculated by superimposing a transparent cellulose acetate sheet with a 1 mm square lattice grid and counting the squares comprised within the outline (each square 1 mm^2).

This methodology was rigorously used in this study.

Quantitative prick by prick procedure, instead, was carried out both with the fresh fennel and microwaved fennel (50 g, 2', 2450 MHz). Microwaving was meant to test the thermostability of fennel proteins. Thus, 50 g of the edible part of fennel were subjected to treatment in a Philips M305 microwave hoven at a 2450 MHz for 2'. The patient's skin was pricked through a thin piece, usually a thin slice, of the fresh food using a sterile pricker needle and, if the

response was positive, the area of the wheals was measured in the same way described for skin prick testing.

Cod	Mussels	Shrimp	Cuttlefish
Mackerel	Egg albumen	Egg yolk	Lacto-albumin
Casein	Beta-lactoglobulin	Wheat grain	Rice flour
Peanuts	Almonds	Hazelnuts	Beans
Peas	Potatoes	Tomatoes	Celery
Fennel	Lettuce	Apricots	Oranges
Bananas	Cherries	Strawberries	Kiwi
Apple	Melon	Peach	Plums
Grapes	Sunflower		

Tab. 3. Collection of 34 commercial food allergen extracts used in skin prick testing.

2.1.3. In vitro tests

Sera from patients with fennel allergy were tested for fennel-specific IgE by ImmunoCAP (Thermo Fisher Scientific, Milan, Italy), according to the instructions of the manufacturer (http://www.phadia.com/it/5/Prodotti/Dosaggi/1/#ImmunoCAP).

2.2. Generation of semi-purified food extracts

2.2.1. Preparation of a semi-purified fennel extract

A suitable amount (100 g) of the edible part of fresh *F. vulgare* was washed properly, minced and homogenated for 15' by a Heidolph DIAX 900 homogenizer with a Heidolph 10 F probe, at 25,000 revolutions per minute, on ice, in the presence of 10 ml PBS 10X w/o Ca^{++} e Mg^{++} (final concentration ~ 1X), 400 µl Ethylenediaminetetraacetic acid (EDTA) 0,5 M (final concentration 2 mM) and 700 µl of plant cell-specific protease inhibitor cocktail (Sigma, Milan, Italy). The inhibitor cocktail contained: 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1,10-phenantroline, pepstatin A, bestatin and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), in unknown concentration. The homogenate was then centrifuged at 12,000 x g, for 20' at 4 °C and the supernatant was recovered. This step was repeated two more times. Successively, the sample was ultracentrifuged at 100,000 x g,

for 2 hs, at 4 °C. Forty ml of 100,000 x g supernatant were obtained from 100 g of fresh *F. vulgare*. The extract was kept at -80 °C, until used.

The protein content, determined according to the colorimetric Bradford method (Bradford, 1976), was 3.5 mg/ml.

2.2.2. Preparation of semi-purified peach and celery 100,000 x g supernatants

A suitable amount (50 g) of the edible portion of fresh *Prunus persica* and *Apium graveolens*, respectively, was washed, minced and homogenated for 15', as above, on ice, in the presence of 5 ml PBS 10X w/o Ca^{++} e Mg^{++}, 200 µl EDTA 0,5 M and 350 µl of plant cell-specific protease inhibitor cocktail, as above. The homogenates underwent three centrifugation cycles at 12,000 x g, for 20' at 4 °C. Successively, the homogenates were subjected to ultracentrifugation at 100,000 x g, for 2 h at 4 °C, thus generating semi-purified 100,000 x g supernatants. The protein content, determined by the colorimetric Bradford method (Bradford, 1976), was 0.228 mg/ml for the peach extract and 0.8 mg/ml for the celery extract. The two extracts were maintained at -80 °C, until needed.

2.3. Biotinylation of the semi-purified fennel extract

Upon thawing, 2 ml of the semi-purified fennel extract, containing 7 mg of total proteins [3.5 mg/ml], were applied to a gel filtration Sephadex G 25 column (PD-10 columns, Amersham Biosciences, Sweden), previously conditioned with 30 ml PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA. The column was eluted with 13 ml PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA. As it is known, gel filtration is a chromatography technique that allows not only separation of biomolecules, such as peptides, proteins, nucleic acids, etc., on the basis of their size, but also to change the composition and pH of the sample buffer. Molecules move through a bed of porous beads, diffusing into the beads to a greater or lesser degree. Smaller molecules enter the pores of the beads and therefore move through the bed slowly, while larger molecules enter less or do not enter at all, moving through the bed quickly. Based on this principle, the elution of the sample through the gel filtration column may also determine the pH shift of the sample of interest.

Thus, 20 fractions (1-20, each of them of about 500 µl) were collected and stored at 4 °C, upon elution of the PD-10 column. The protein amount of each fraction was determined by the colorimetric Bradford method.

Fractions from 1 to 5 had a protein concentration below 0.1 mg/ml and were discarded.

Fractions from 10 to 20, with a protein concentration between 0.1 mg/ml and 0.6 mg/ml, were pooled, yielding an approximately 6 ml sample. This sample was concentrated, using 3 Centricon ultrafiltration devices (2 ml were loaded in each device), with a cut-off of 10,000 Da (Amicon Ultra-4, Millipore, Billerica, U.S.A.). The devices were then subjected to centrifugation at 7,500 x g for 20', at 4 °C. Ultrafiltration by these devices allows recovery of a retentate containing molecules > 10,000 Da and an ultrafiltrate with molecules < 10,000 Da. The protein content of each of the two fractions was determined by the colorimetric Bradford method. The retentate, with a volume of 450 µl, had a protein concentration of 4.6 mg/ml, while protein concentration of the ultrafiltrate (about 4 ml) was below the method sensitivity. Pool of fractions 6-9 (about 2 ml), containing most of the proteins, instead, had a protein content of 5.2 mg. This pool was biotinylated using 1.8 mg of biotin disulphide N-hydroxysuccinimide ester (Berry & Associates, Dexter, U.S.A). The reagent included in the reaction was proportional to the mass of the proteins to be biotinylated, according to the following formula: mg of reagent = mg protein to be biotinylated x 20 x 341.39 / 20,000, where 20 is the molar excess of the reagent (between 10 and 30), 341.39 is the biotin molecular weight and 20,000 is the estimated average molecular weight of the proteins to be biotinylated. Biotin disulphide N-hydroxysuccinimide ester was dissolved in dimethylformamide (1.8 mg in 100 µl) and an appropriate volume of this solution (72 µl) was added to pool 6-9. The reaction sample was gently mixed for 1 h at room temperature. In order to remove the biotin unbound to proteins of interest, the biotinylated sample was subjected to gel filtration on a column of Sephadex G 25 (PD-10) that had previously been conditioned with 30 ml PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA. Therefore, the sample was eluted with 13 ml PBS w/o Ca^{++} e Mg^{++}1 mM EDTA. Twenty-three fractions (1-23, each of them with a volume of about 500 µl) were collected and their protein amount was determined by the Bradford method. The vast majority of the biotinylated fennel sample was present in pool 6-9. The protein concentration of this pool was 1.9 mg/ml. This biotinylated fennel extract was used in an ELISA *in vitro* assay, by employing, in part, a commercial kit marketed by RADIM (Pomezia, Rome).

2.4. The ELISA capture *in vitro* assay

To perform the in-house RAST-capture assay, an ELISA 96-wells microplate, with anti-IgE antibodies coated on the surface of wells, was used (RADIM, Pomezia, Rome). The wells were first blocked with a blocking solution made of 10% non-fat dried milk in PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA (350 µl/well were dispensed) and incubated at 37 °C overnight. The next day, the blocking solution was removed and the IgE microplate was processed using a commercial kit, also marketed by RADIM, including all the necessary reagents.

In the microplate, two blanks were set up and six wells were used for human IgE calibrators, with the following concentrations: 0.36, 0.72, 3.6, 18, 50 and 100 kU/l. The calibrators were needed to draw the calibration curve.

The protocol used was as follows:

step 1: 50 µl of incubation buffer were first dispensed into all the wells;

step 2: 50 µl of each of the calibrators or 50 µl of each of the samples were loaded in the respective wells; instead, other 50 µl of incubation buffer were loaded into the blanks;

step 3: the microplate was incubated for 60 ± 5', at 37 °C;

step 4: the microplate was washed 3 times (the working solution was preparared from a 10X solution, provided with the kit);

step 5: either 100 µl of anti-IgE biotin conjugated antibody or 100 µl of the biotin conjugated fennel allergen, respectively, were dispensed into the calibrator wells and the sample wells, respectively. The biotinylated allergen amount selected as suitable for the assay was 1 µg per well. Since the protein concentration of the biotinylated fennel extract was 1.9 mg/ml and the volume of the biotinylated allergen solution to be used in the test was 100 µl per well, a 1:200 dilution of the biotinylated fennel extract was performed. Hundred µl of this diluted solution were added to the relevant wells (containing ~ 1 µg of the biotinylated fennel extract);

step 6: the microplate was then incubated for 30 ± 5', at 37 °C;

step 7: then it was washed again 3 times;

step 8: 100 µl of HRP (horseradish peroxidase)-streptavidin conjugated were added to all the wells;

step 9: the microplate was incubated for 30 ± 5', at 37 °C;

step 10: the wells were washed for 3 times;

step 11: 100 µl of HS (High Sensitivity) substrate were added to all the wells;

step 12: the microplate was incubate for 15 ± 1' at room temperature, in the dark;

step 13: finally, 100 µl of stop solution were dispensed in all the wells;
step 14: the resulting colorimetric reaction intensity was read at 450 and 405 nm with the reference filter set at 595 nm in a Microplate Reader (model 450, Bio-Rad). The reaction was read at 405 nm, when the absorbance at 450 nm was above 2.0 O.D. IgE title of the samples, expressed as kU/l, was determined by interpolation on the calibration curve.

2.5. RAST inhibition experiments

RAST inhibition experiments were performed to evaluate the possible cross-reactivity between fennel and peach extracts and between fennel and celery extracts. RAST inhibition experiments were also performed between the semi-purified fennel extract and biotinylated fennel allergen in order to validate the results obtained with the other two settings above.
Regarding the peach RAST inhibition experiments, 50 µl of serum from 4 fennel allergy patients (B.L., F.M., P.A. and P.G.; see below the Results section) were pre-incubated for 24 h at 4 °C, while gently shaking, with peach extract in PBS w/o Ca^{++} e Mg^{++} 2.5 mM EDTA with 4 different amounts of peach proteins, *i.e.*, 0 µg, 0.228 µg, 1.14 µg and 4.5 µg (4 samples for each of the 4 patients). In particular, the following volumes of the original peach 100,000 x g supernatant [0.228 mg/ml] included in the buffer mentioned above, up to a final total volume of ~ 50 µl were: 0 µl, 1 µl, 5 µl and 20 µl.
The celery RAST inhibition experiments were performed following the same protocol as above. However, the celery protein content of the 4 samples generated for each of the same 4 patients as above, were 0 µg, 0.8 µg, 1.6 µg and 8 µg. In particular, the following volumes of the original celery 100,000 x g supernatant [0.8 mg/ml] were included in the buffer mentioned above, up to a final total volume of ~ 50 µl: 0 µl, 1 µl, 2 µl and 10 µl.
Finally, for the fennel RAST inhibition experiments, sera from the 4 patients with fennel allergy were pre-incubated in the presence of 0 µg, 0.35 µg, 1.05 µg and 10.5 µg of fennel proteins. In this case, the volumes of the original fennel 100,000 x g supernatant [3.5 mg/ml] were included in the buffer mentioned above, up to a final total volume of ~ 50 µl: 0 µl, 1 µl and 3 µl from a dilution 1:10 of the semi-purified fennel extract, respectively and 3 µl from the undiluted fennel extract.

As in the ELISA-capture assay, a 96-wells microplate, with anti-IgE antibodies coated on the surface of wells, was used (from RADIM). The wells were first blocked with a blocking solution made of 10% non-fat dried milk in PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA (350 µl/well) and incubated at 37 °C overnight. The next day, the pre-incubated samples were centrifuged at 20,000 x g for 30' at 4 °C. Upon removal of the blocking solution from the wells, 50 µl of the supernatant of the pre-incubated samples (after centrifugation) were added to each well. Successively, the IgE microplate was processed following the instructions of the commercial kit marked by RADIM, as described above.

2.6. Immunodetection of fennel allergens

2.6.1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the semi-purified *Foeniculum vulgare* extract

The in-house fennel extract was analyzed by SDS-PAGE. Upon thawing, SDS-PAGE samples were prepared, with a 12.5 µl volume of fennel extract, corresponding to a protein content of about 45 µg (the protein concentration of the semi-purified *F. vulgare* extract was 3.5 mg/ml), included in a 12.5 µl volume of 2X Laemli loading buffer (125 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-β-mercaptoethanol, 0.02% bromophenol blue). The samples were boiled at 100 ºC for 5', chilled on ice for 5' and then briefly centrifuged, at 20,000 x g. Moreover, prestained SDS-PAGE Standards – low range samples, with known molecular weight (Bio-Rad, Richmond, CA) were prepared by adding 10 µl of these markers to an equal volume of 1X Laemli loading buffer (62.5 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol, 0.01% bromophenol blue). Unlike fennel extract samples, standards were unboiled. Other prestained SDS-PAGE standards were also used in selected experiments: Kaleidoscope Prestained Standards (Bio-Rad, Richmond, CA) and Precision Plus Protein Dual Color Standards (Bio-Rad, Richmond, CA).

Protein bands were separated in a continuous gel. SDS-PAGE was performed on 1.5 mm slab gels (10% resolving gel and 5% stacking gel). Acrylamide/bis-acrylamide mixture with a ratio 29:1 was used (Sigma, Milan, Italy).

Resolving gel and stacking gel solutions were prepared as follows. The 10% resolving gel solution was made of 5 ml acrylamide/bis-acrylamide mixture with a ratio 29:1, 6.8 ml

Millipore water, 3 ml Tris HCl 1.5 M pH 8.8, 150 µl 10% Sodium Dodecyl Sulphate (SDS), 15 µl Tetramethylethylenediamine (TEMED; Bio-Rad, Richmond, CA) and 100 µl Ammonium Persulfate (APS; Sigma, Milan, Italy). The 5% stacking gel solution was prepared with 1.62 ml acrylamide/bis-acrylamide mixture, 5.8 ml Millipore water, 2.5 ml Tris HCl 0.5 M pH 6.8, 100 µl 10% SDS, 15 µl TEMED and 100 µl APS.

The run was carried out at 80 V for 3 h on a refrigerated Mini-Protean II vertical electrophoresis apparatus (Bio-Rad, Richmond, CA).

2.6.2. IgE Immunoblotting analysis

After electrophoresis, the separated proteins were electro-transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire UK), using a Mini Transphor TE22 (Hoefer Scientific Instrument, San Francisco U.S.A), at 200 mA for 2 h at 4 ºC.

After electroblotting, the gel was stained with 0.1% Coomassie Brilliant Blue G 250 (Merck, Darmstadt, Germany) in water:methanol:acetic acid (50:40:10), overnight at room temperature and, successively, rinsed in water:methanol:acetic acid (50:40:10) destaining solution, three times for 20', at room temperature. Finally, it was dried with a gel-dryer (Drygel Sr., Slab Gel Dryer, Hoefer Scientific Instrument, San Francisco U.S.A), in order to assess transfer efficiency.

The blotted nitrocellulose membrane was subjected to blocking of the non-specific binding sites (5% non-fat dried milk in 50 mM TBS, pH 7.5), for 1 h at room temperature. Then the membrane was cut into strips, each corresponding to a gel lane. The strips were incubated overnight at 4 ºC, while rotating (11 rpm) with 1 ml of serum from a fennel allergic patient (the primary antibody), diluted 1:5 in 50 mM TBS pH 7.5, with 5% inactivated fetal calf serum (FCS). Alternatively, the strips were incubated with serum from control subjects.

Successively, strips were rinsed in 50 mM TBS pH 7.5 with the addition of 0.1% Nonidet NP40, 2 times for 10', at room temperature. Typically, the strips were re-incubated with 2 ml of an anti-human IgE peroxidase-conjugated polyclonal antibody, raised in goat (Sigma, Milan, Italy), diluted 1:330 in 50 mM TBS pH 7.5, in the presence of 5% non-fat dried milk, for 3 h at room temperature, on rotator (11 rpm). Alternatively, an anti-human IgE peroxidase-conjugated monoclonal antibody (Abcam, Cambridge, UK) was also adopted in selected experiments. After incubation with the secondary antibody, each strip was rinsed in 50 mM TBS pH 7.5 added to 0.1% Nonidet NP40, 3 times for 10' at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL, GE

Healthcare, Buckinghamshire UK), according to the manufacturer instructions. In particular, the volume of the ECL reagent (in ml) was given by the membrane area (in cm^2) x 0.125. The ECL signal was recorded by exposing pre-flashed RX films (AGFA Healthcare, Belgium), typically for 15'. Shorter (down to 30'') and longer (up to 1 h) exposure times were also adopted, occasionally.

Finally, the immunoreactive bands molecular weight was inferred by comparison with known molecular weight markers, loaded in the same gel of fennel extract samples (Damiani et al., 2012; De Cesare et al., 1993; Di Felice et al., 1994).

2.7. Statistical analysis

Multiple observations were reported as the arithmetical mean ± standard deviation. Comparison between different populations of data was performed according to Mann-Whitney Rank Sum Test for independent samples.

Correlation analysis was carried out to associate the RAST-capture assay results with that obtained by the automated ImmunoCAP Thermo Fisher system, allowing calculation of Pearson's linear correlation coefficient.

3. Results

3.1. Selection of study patients

A group of 189 essentially adult patients with food allergy diagnosis consecutively, observed at our outpatient clinic between 1st February 2013 and 28th June 2013, was screened for fennel allergy. This group included 71 males and 118 females, with a mean age of 31.3 ± 13.2 (Tab. 4.).

	Population	N patients	Males	Females	Minimum age	Maximum age	Mean age	Standard deviation
A	Patients with food allergy	**189**	71	118	5	77	31.3	13.2
B	Patients with non-symptomatic cutaneous positivity to fennel	**24**	13	11	16	60	28.7	11.2
C	Patients with fennel allergy	**57**	27	30	12	58	29.3	9.9
D	Patients with fennel allergy studied	**44**	21	23	12	58	30.6	10.8

Tab. 4. Demographic features of: A) 189 food allergy patients screened; B) 24 patients with cutaneous positivity to fennel only; C) 57 patients with fennel allergy; D) 44 patients subjected to the fennel allergy study protocol.

The analysis of these 189 food allergy patients revealed that 81 of them (43%) showed positive SPT for fennel, indicating the presence of fennel-specific mast cell-bound IgE.

In particular, among this population, 24 patients were sensitized to fennel proteins, but they did not report symptoms associated with fennel consumption. This sub-group included 13 males and 11 females, with a mean age of 28.7 ± 11.2 (Tab. 4.).

Moreover, SPT carried out with a commercial fennel extract marketed by Lofarma (Milan) showed an average wheal area, expressed in mm^2, equal to 15 ± 10 mm^2, in this sub-group (Tab. 5.).

Patient	*Wheal area (mm^2)*
B.V.	5
C.R.L.	8
C.V.	11
C.V.	33
D.M.A.	9
D.P.G.	14
D.K.	21
G.R.	45
K.A.	6
L.C.	14
L.F.A.	12
M.V.	6
M.D.	14
P.A.	3
P.C.	21
R.M.	8
S.M.	15
S.L.	29
S.C.	22
S.G.	10
S.M.	25
S.H.	7
S.A.	18
S.D.	9
Average area of the wheals	**15**
Standard deviation	**10**

Tab. 5. SPT carried out with a commercial fennel extract marketed by Lofarma (Milan). The results shown are expressed as areas of the wheals (mm^2) (n=24 patients).

Moreover, among the 189 food allergy patients, fennel allergy was diagnosed in 57 patients (30%). This sub-group of fennel allergy patients included 27 males and 30 females, with a mean age of 29.3 ± 9.9 (Tab. 4.).

In all these 57 patients occurrence of fennel-specific IgE had been detected by routine skin prick testing with commercial extracts marketed by Lofarma (Milan) (the complete list of food allergens employed for routine skin testing is reported in the Materials and Methods section) and, in certain cases (n=5 patients), by prick by prick with fresh fennel. The results of these *in vivo* investigations, expressed as average wheal area elicited in mm^2, were 20 ± 15 mm^2 for skin prick testing and 26 ± 13 mm^2 for prick by prick tests (Tab. 6.).

Patient	*Wheal area (mm^2)*	
	Commercial extract	*Raw fennel*
B.A.	10	
B.G.	38	
B.L.	25	
C.A.	36	
C.C.	12	
C.G.	4	
C.R.	6	
C.G.	7	
C.M.	8	
C.A.R.	10	
C.A.	28	
C.G.	10	
C.M.	22	39
D.A.G.	17	
D.B.A.	25	
D.V.M.	11	
D.A.	31	
D.B.A.	no data	41
D.C.A.	27	
D.V.L.	85	
D.F.	19	
F.M.	12	
F.F.	23	
L.A.	0	
L.V.	41	
L.V.	11	
L.C.	no data	
L.A.	15	
M.D.	19	
M.V.	9	
M.F.	9	
M.C.	15	
M.G.	no data	13
M.A.	29	
M.D.P.	46	
M.F.	14	
M.B.	11	
M.D.	45	
M.R.	11	
P.A.	7	
P.T.	28	
P.A.	23	
P.R.	17	
P.G.	6	
R.A.	36	

R.G.	6	
R.A.	35	
R.M.L.	12	
S.V.	19	
S.G.	6	15
S.G.D.	11	
S.N.	37	
S.R.	20	
T.G.	40	
V.V.	3	23
V.D.C.	no data	
V.R.	14	
Average area of the wheals	**20**	**26**
Standard deviation	**15**	**13**

Tab. 6. Routine SPT performed with a commercial fennel extract marketed by Lofarma (Milan) and prick by prick tests (in 5 patients only) carried out using raw fresh fennel. In both cases, results are expressed as areas of the wheals (mm^2) (n=57 patients).

As regards the clinical history, at the routine visit, all 57 patients had reported symptoms associated with fennel consumption consistent with the diagnosis of fennel allergy.

Fourteen of these fennel positive patients had no other associated food sensitizations, detectable by the routine skin testing procedure adopted. Whereas the remaining 43 patients showed 601 associated sensitizations to other food allergens.

The most frequent associated sensitizations (occurrence > 50%), as detected by SPT, were:

- peanuts: (53/57 patients; 93%);
- hazelnuts: (49/57 patients; 86%);
- beans: (45/57 patients; 79%);
- peach: (45/57 patients; 79%);
- peas: (42/57 patients; 74%);
- wheat grain: (36/57 patients; 63%);
- rice flour: (36/57 patients; 63%);
- apple: (31/57 patients; 54%).

See also Tab. 7.

Thus, based on the simultaneous occurrence of a convincing clinical hystory and detection of specific IgE *in vivo*, the diagnosis of fennel allergy was put forward in all of these 57 patients.

Food allergens	*Occurrence*	*(%)*
Peanuts	53	93
Hazelnuts	49	86
Beans	45	79
Peach	45	79
Peas	42	74
Wheat grain	36	63
Rice flour	36	63
Apple	31	54
Almonds	27	47
Walnuts	25	44
Corn	22	39
Tomatoes	20	35
Celery	20	35
Soy	20	35
Oranges	18	31
Cherries	17	30
Lettuce	14	25
Plums	14	25
Strawberries	10	17
Melon	8	14
Potatoes	7	12
Sunflower	6	10
Norway lobster	5	9
Bananas	5	9
Apricots	4	7
Kiwi	4	7
Egg albumen	3	5
Yeast	3	5
Cod	2	3
Egg yolk	2	3
Mussels	1	2
Grapes	1	2
Artichoke	1	2
Rabbit	1	2
Lentils	1	2
Pork	1	2
Mustards	1	2
Tuna	1	2

Tab. 7. Accociated cutaneous sensitizations to other, then fennel, food allergens (n=43 patients).

SPT using inhalant allergens were also carried out in the 57 patients with fennel allergy and the occurrence was calculated. As it is possible to observe in the table below (Tab. 8.), the most frequent cutaneous sensitization is represented by that to Artemisia (35 out of 57 fennel allergy subjects) with an occurrence of 61%. Other major respiratory allergic sensitizations were characterized by an occurrence slightly lower than 50%:

- *Parietaria judaica*: (28/57 patients; 49%);
- *Olea europea*: (28/57 patients; 49%);
- *Graminaceae spp.*: (26/57 patients; 46%);
- *Cupressus arizonica*: (26/57 patients; 46%).

Inhalant allergens	*Occurrence*	*(%)*
Artemisia	35	61
Parietaria judaica	28	49
Olea europaea	28	49
Graminaceae	26	46
Cupressus arizonica	26	46
Dog epithelium	22	39
Dermatophagoides pteronyssinus	21	37
Cat epithelium	20	35
Dermatophagoides farinae	15	26
Fraxinus	12	21
Horse	12	21
Platanus	8	14
Quercus ilex (holm)	6	10
Betula	5	9
Corylus avellana (hazel)	5	9
Cladosporium	4	7
Aspergillus	3	5
Alternaria	3	5
Acacia dealbata (mimosa)	3	5
Cupressus sempervirens	2	3
Leucanthemum vulgare (marguerite)	2	3
Amaranth	1	2
Ambrosia	1	2
Rabbit	1	2
Dahlia	1	2
Taraxacum officinale	1	2
Solidago virgaurea	1	2

Tab. 8. Multiple cutaneous sensitizations to inhalant allergens (n=43 patients).

These 57 selected patients were invited to take part into a focused study, aimed at characterizing fennel allergy in detail. The study protocol included:

- a visit with a thorough revision of the clinical history;
- precision skin prick testing. In particular, they consisted in quantitative SPT with a commercial extract marketed by Lofarma and with an in-house semi-purified extract, respectively. Moreover, quantitative prick by prick tests with raw and microwaved fennel, respectively, were performed;
- a blood sample collection. Sera would then be stored at -30 °C to be used later for *in vitro* investigations.

As regards the *in vitro* tests, patiens'sera were analyzed using ImmunoCAP Thermo Fisher system and a RAST-capture assay developed in our laboratory.
Among these 57 patients, 44 complied to participate in the study. These subjects included 21 males and 23 females, with a mean age of 30.6 ± 10.8 (Tab. 4.).

3.2. Generation of a semi-purified fennel extract

In order to carry out the various *in vivo* and *in vitro* studies planned, respectively, an in-house semi-purified fennel extract was generated.
Thus, starting from a suitable amount (100 g) of the edible part of fresh fennel, upon homogenation and exhaustive centrifugation (3 times), to separate the fibrous part of the homogenate (pellet) from the soluble proteins (supernatant), followed by ultracentrifugation, a semi-purified fennel extract was produced. The protein content, determined according to the Bradford method, was 3.5 mg/ml.
Successively, the protein profile of fennel extract was analyzed by SDS-PAGE under reducing conditions. Two different resolving gel composition were used: 10% and 12%, respectively, with a 5% stacking gel in both cases. After electrophoresis, 18 fennel extract protein bands, separated according to their molecular weight, were detected. Moreover, by loading a range of extract protein amounts of this fennel extract (from 4.5 µg to 45 µg) we showed that the band resolution was excellent also when the highest amount (45 µg) was analyzed (Fig. 8.).

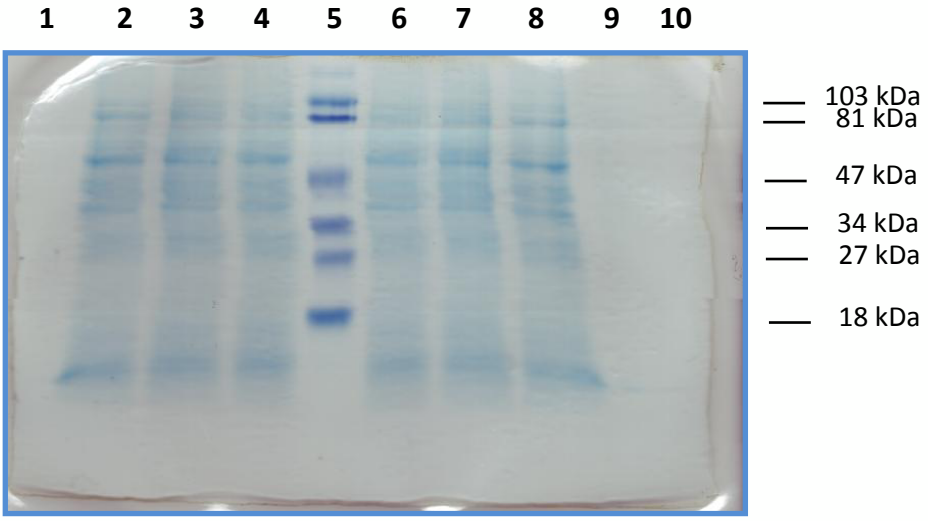

Fig. 8. SDS-PAGE analysis of fennel extract. 45 µg of the semi-purified fennel extract were loaded in lanes 2, 3, 4, 6, 7 and 8. In lane 5 prestained SDS-PAGE standards - low range (Bio-Rad, Richmond, CA) were loaded. The relevant calibrated molecular weights are indicated.

3.3. Clinical features of the selected fennel allergy population

In Tab. 9. the main symptoms and associated diagnoses reported by the patients upon fennel consumption are listed. Thus, 40 out of the 44 fennel allergy patients (91%) reported lip angioedema and oral itching after fennel's ingestion (oral allergy syndrome). Thirteen (29%) lamented cutaneous itching, of which 9 localized to neck, ears or face and 4 generalized, scattered itching in other parts of the body. Moreover, 9 patients (20%) had urticaria; 16 patients (36%) Quincke's edema; 22 patients (50%) respiratory symptoms (in particular, 16 fennel allergy patients reported dyspnea; 3 subjects lamented chest tightness; 2 patients reported wheezings and 1 coughing); 14 patients (32%) gastrointestinal symptoms (such as nausea, vomiting, epigastric and abdominal pains, diarrhea) and, finally, two patients (4%) experienced severe anaphylaxis, after eating raw fennel (Tab. 9). Moreover, 3 patients (7%) stated that they had never eaten fennel, because they disliked it or even felt repulsion if one just mentioned this vegetable.

Symptoms	N patients	(%)
Oral allergy syndrome	40	91
Itching	13	29
Urticaria	9	20
Quincke's edema	16	36
Bronchospasm	22	50
Gastrointestinal symptoms	14	32
Anaphylaxis	2	4
No reactions*	3*	7

Tab. 9. Symptoms observed in 44 patients studied for fennel allergy. *These 3 patients stated that they had never eaten fennel, because of repulsion toward this vegetable.

3.4. Studies on mast cell-bound specific IgE by precision skin prick tests and prick by prick

The 44 patients who participated in the fennel allergy study were subjected to SPT and prick by prick procedure, in order to detect mast cell-bound specific IgE for fennel. However, 2 out of these 44 fennel allergy patients (F.M. and P.A.), at the time of the visit, declined undergoing the *in vivo* tests.

Quantitative SPT were carried out using a commercial extract marketed by Lofarma (Milan) (concentration unknown) and the semi-purified in-house fennel extract (with a protein concentration of 3.5 mg/ml), respectively. The results of these tests were expressed as areas of the wheals elicited (mm^2), see Tab. 10. As it is known, the magnitude of wheal response is proportional to the amount of endogenous histamine released by cutaneous mast cells, activated as a consequence of the test, which, in turn, reflects the amount of the existing specific IgE linked to mast cell surface by the high affinity receptor FcεRI.

It is also worth recalling that the relation between FcεRI-bound specific IgE and size of the wheal elicited by skin prick test is essentially logarithmic.

Patient	*Wheal area (mm^2)*			
	Commercial extract [1]	*Fennel extract* [1]	*Raw fennel* [2]	*Microwaved fennel* [2]
B.G.	52	134	50	83
B.L.	48	85	27	57
C.A.	81	48	27	50
C.C.	24	67	45	27
C.R.	7	8	10	8
C.M.	10	28	24	0
C.A.R.	18	27	24	15
C.A.	14	40	39	97
C.M.	18	60	39	29
D.A.G.	5	27	16	7
D.V.M.	9	48	40	22
D.A.	25	38	18	22
D.B.A.	48	96	254	158
D.C.A.	11	60	64	76
D.V.L.	30	241	35	64
D.F.	17	20	19	8
F.M.*	no data	no data	no data	no data
L.A.	8	12	29	7
L.V.	34	74	32	60
L.V.	24	50	49	46
M.D.	28	68	56	24
M.C.	12	20	27	26
M.G.	33	42	38	29
M.A.	53	77	57	16
M.D.P.	88	54	60	103
M.B.	17	18	16	6
M.D.	33	66	12	11
M.R.	19	22	27	27
P.A.	11	14	17	0
P.T.	33	197	117	42
P.A.*	no data	no data	no data	no data
P.R.	11	99	15	44
P.G.	15	65	140	27
R.A.	41	30	23	49
R.G.	28	42	42	47
R.M.L.	26	45	17	13
S.V.	26	46	22	20
S.G.	8	14	15	17
S.N.	10	36	43	24
S.R.	11	39	10	18
T.G.	31	80	22	36
V.V.	16	17	16	5
V.D.C.	23	121	88	46
V.R.	21	110	39	38

Average area of the wheals	26	59	42	36
Standard deviation	18	47	43	32

Tab. 10. 1: SPT carried out with a commercial fennel extract marketed by Lofarma (Milan) and the semi-purified in-house *F. vulgare* extract, respectively. 2: Prick by prick tests performed with raw fennel and microwaved fennel, respectively. In both cases, results are expressed as areas of the wheals elicited in mm^2 (n=42 patients). *These two fennel allergy patients declined undergoing the *in vivo* tests.

Statistical analysis performed according to the Mann-Whitney Rank Sum Test revealed that the difference observed between the average area of the skin prick test wheals obtained with the commercial extract (26 ± 18 mm^2) and the average area of those obtained using the in-house *F. vulgare* extract (59 ± 47 mm^2) was statistically significant ($p < 0.001$).

Moreover, in these 44 patients we performed quantitative prick by prick tests with raw fennel and microwaved fennel (2450 MHz for 2'). The results (areas in mm^2) are shown in Tab. 10. It is worth noticing that, in this case, the average area of the prick by prick wheals (mm^2) obtained with raw fennel (42 ± 43 mm^2) did not significantly differ from that obtained with microwaved fennel (36 ± 32 mm^2), according to the Mann-Whitney Rank Sum Test ($p=0.562$).

Many of 44 patients with fennel allergy also exhibited multiple sensitizations to other food allergens belonging to the *Apiaceae* family. Thus, 29 patients (69%) had positive SPT for celery (*A. graveolens*), 11 (26%) for carrot (*Daucus carota*) and 37 (88%) for parsley (*Petroselinum crispum*; Fig. 9). In the latter case, prick by prick tests were performed with the fresh vegetable, because a commercial extract of parsley was not available (unlike celery and carrot).

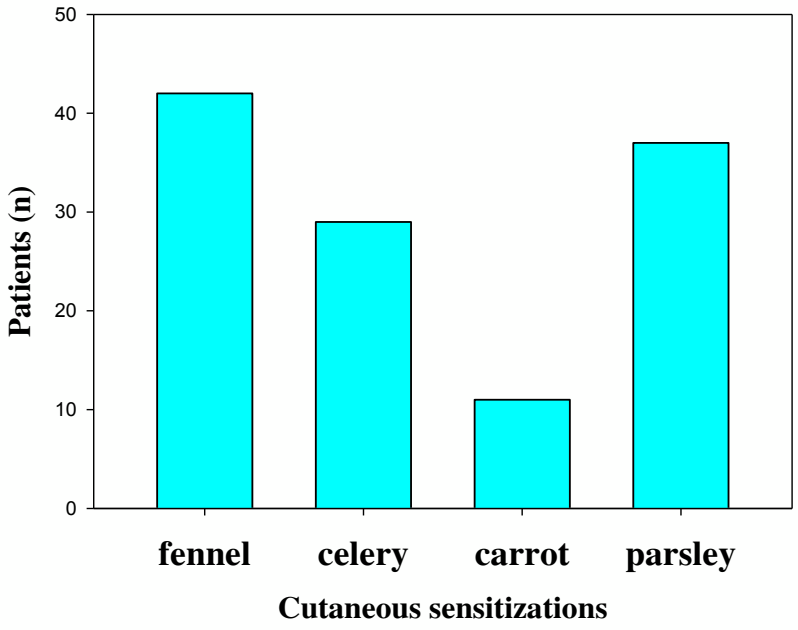

Fig. 9. Allergic sensitizations to other food allergens belonging to the *Apiaceae* family (n=42 patients). SPT with fennel, celery and carrot commercial extracts, respectively were performed. Only in the case of parsley, prick by prick tests were performed, because no parsley commercial extract was available.

Moreover, wheal areas obtained in SPT, carried out with celery and carrot commercial extracts, marked by Lofarma (Milan) and in prick by prick tests, performed using fresh parsley, were calculated (Tab. 11.).

Patient	*Wheal area (mm^2)*		
	Celery extract	*Carrot extract*	*Parsley**
B.G.	15	0	37
B.L.	22	10	0
C.A.	11	0	52
C.C.	17	0	22
C.R.	6	0	0
C.M.	13	0	0
C.A.R.	15	15	12
C.A.	9	0	60
C.M.	11	8	19
D.A.G.	0	0	6
D.V.M.	10	0	0
D.A.	0	0	10
D.B.A.	11	0	36
D.C.A.	13	0	25
D.V.L.	18	0	22
D.F.	23	0	8
F.M.*	no data	no data	no data
L.A.	0	0	21
L.V.	0	0	14
L.V.	0	0	19
M.D.	12	0	33
M.C.	0	0	4
M.G.	11	0	11
M.A.	13	7	16
M.D.P.	9	8	120
M.B.	21	3	5
M.D.	12	0	17
M.R.	0	13	19
P.A.	9	0	6
P.T.	10	0	33
P.A.*	no data	no data	no data
P.R.	0	0	10
P.G.	10	0	14
R.A.	9	0	8
R.G.	0	0	13
R.M.L.	0	0	16
S.V.	15	0	6
S.G.	9	8	21
S.N.	10	0	7
S.R.	9	11	19
T.G.	12	0	14
V.V.	0	0	0
V.D.C.	17	0	42
V.R.	0	0	35

Average area of the wheals	9	2	20
Standard deviation	7	4	21

Tab. 11. SPT carried out with celery and carrot commercial extracts marketed by Lofarma (Milan), respectively. *In this case, prick by prick tests were performed using fresh parsley, because no parsley commercial extract was available. In both cases, results are expressed as areas of the wheals (mm^2). N=42 patients. *These 2 fennel allergy patients refused to repeat the *in vivo* tests.

Associated multiple sensitizations to other allergens (not belonging to the *Apiaceae* family) were also analyzed in these 44 fennel allergy patients. Thus, SPT with the 9 food allergens most frequently associated with fennel sensitization were performed. The 9 food allergens were: peanuts, beans, hazelnuts, peas, apple, peach, almonds, rice flour and wheat grain. These allergens had been selected on the basis of their rate of co-occurrence with fennel sensitization (> 50% for all of them, except almonds: 47%) as assessed by the analysis of the data obtained by routine skin testing in the 57 fennel allergy patients described above.

The results of these investigations are reported in Tab. 12. As it can be seen, all patients (42 out of 42, because among the 44 fennel allergy patients recruited 2 had declined undergoing the *in vivo* tests) had a positive shin prick test for peanuts, 40 patients (95%) for beans, 39 patients (93%) for hazelnuts, 37 patients (88%) for peas, 35 patients (83%) for apple and peach, 33 patients (79%) for almonds, 32 patients (76%) for rice flour and, finally, 30 patients (71%) for wheat grain. Also in Tab. 11, the number of patients sensitized to the 9 food allergens, respectively, and lamenting symptoms clearly associated with consumption of the relevant food are reported.

Food allergen	*Positive patients (n)*	*% [1]*	*Patients with relevant symptoms (n)*	*% [2]*
Peanuts	42	100	32	76
Beans	40	95	9	22
Hazelnuts	39	93	30	77
Peas	37	88	14	38
Apple	35	83	13	37
Peach	35	83	33	94
Almonds	33	79	18	54
Rice flour	32	76	5	16
Wheat grain	30	71	1	3

Tab. 12. Multiple cutaneous sensitizations to other important food allergens in n=42 patients with fennel allergy. The number of allergic patients with clinically significant sensitization is also reported. %[1]: positive patients out of the 42 patients with fennel allergy. %[2]: patients with clearly associated symptoms out of those who were sensitized.

Moreover, the areas of the wheals elicited (mm^2) in quantitative skin prick testing, performed using the commercial extracts (marketed by Lofarma, Milan) of the 9 food allergens more frequently associated with fennel sensitization were measured. The results of these tests are shown in Tab. 13.

Patient	*Wheal area (mm^2)*								
	Peanuts	*Beans*	*Hazelnuts*	*Peas*	*Apple*	*Peach*	*Almonds*	*Rice flour*	*Wheat grain*
B.G.	70	86	46	43	54	80	7	29	16
B.L.	41	49	53	110	29	22	43	41	26
C.A.	33	19	57	24	35	30	7	0	0
C.C.	34	23	29	30	23	17	0	11	16
C.R.	7	5	4	0	0	0	0	0	0
C.M.	15	9	0	15	0	0	0	4	10
C.A.R.	42	43	16	28	25	25	25	17	14
C.A.	48	30	36	34	16	49	5	10	6
C.M.	18	43	16	17	14	16	10	16	17
D.A.G.	23	14	7	16	9	5	6	0	0
D.V.M.	37	31	12	25	17	24	21	9	7
D.A.	4	16	18	0	0	0	0	0	0
D.B.A.	33	31	41	29	18	24	6	20	18
D.C.A.	18	24	13	17	14	0	8	13	0
D.V.L.	212	22	47	192	73	70	29	17	16
D.F.	16	17	10	14	9	18	10	8	9
F.M.*	no data	no data	no data	no data	no data	no data	no data	no data	no data
L.A.	20	0	0	6	0	0	0	0	50
L.V.	10	10	11	8	29	32	0	10	7
L.V.	60	36	56	44	16	35	17	0	0
M.D.	60	41	32	21	20	18	6	18	14
M.C.	16	8	30	8	0	0	0	7	0
M.G.	35	10	29	24	23	28	18	11	10
M.A.	49	24	38	23	13	18	22	15	12
M.D.P.	64	6	29	0	65	82	20	9	6
M.B.	7	6	18	0	11	4	10	0	0
M.D.	24	27	23	19	37	33	15	16	13
M.R.	25	26	24	26	29	32	29	15	19
P.A.	6	12	7	14	0	5	0	0	16
P.T.	44	29	60	41	26	27	28	23	20
P.A.*	no data	no data	no data	no data	no data	no data	no data	no data	no data
P.R.	29	31	9	24	8	20	4	10	0
P.G.	9	16	39	16	11	28	12	8	11
R.A.	63	34	64	100	21	36	24	16	17
R.G.	26	34	28	19	11	20	10	9	11
R.M.L.	16	20	22	30	22	39	11	20	0
S.V.	15	9	22	16	9	11	9	11	29
S.G.	11	10	10	9	9	8	12	12	7
S.N.	50	27	30	40	21	22	9	24	14
S.R.	45	18	31	34	11	11	14	12	19
T.G.	18	19	16	15	15	0	5	13	10
V.V.	9	0	0	0	0	0	0	0	0
V.D.C.	41	38	38	26	32	42	18	19	12
V.R.	21	12	25	36	28	50	11	0	0

Average of wheal area	34	23	26	28	19	23	11	11	11
Standard deviation	33	16	17	34	16	21	10	9	10

Tab. 13. Skin prick testing performed with the commercial extracts (marketed by Lofarma, Milan) of the 9 food allergens more frequently associated with fennel sensitization (peanuts, beans, hazelnuts, peas, apple, peach, almonds, rice flour and wheat grain), respectively. The results are expressed as areas of the wheals elicited in mm^2 (n=42 patients). * These 2 patients with fennel allergy declined complying with the procedure.

In this study, 14 non-allergic subjects, used as control negative, were also recruited. They were subjected to the same *in vivo* protocol of 44 patients with fennel allergy diagnosis and the results were negative.

3.5. *In vitro* studies: ImmunoCAP Thermo Fisher analysis and development of an in-house RAST-capture assay

3.5.1. ImmunoCAP Thermo Fisher analysis of sera from 44 fennel allergy patients

RAST is considered a second level exam in the routine diagnosis of allergic diseases. This technique allows determination of specific IgE in serum and other biological fluids.

We first analyzed the sera obtained from the 44 patients participating in the fennel allergy study for specific IgE toward fennel by the commercially available ImmunoCAP RAST, marketed by Thermo Fisher. Fennel-specific IgE assessment was carried out according to the manufacturer instructions. The results of ImmunoCAP analysis, expressed as kU/l, are reported in Tab. 14. According to the method specifications, IgE values > 0.1 kU/l were considered positive. Therefore, 40 (91%) out of the 44 patients' sera examined were considered positive. Only four patients were RAST-negative.

Patient	*IgE Title (kU/l)* [1]	*IgE Title (kU/l)* [2]
B.G.	2.30	0.20
B.L.	8.97	12.2
C.A.	1.28	0.15
C.C.	5.78	13.4
C.R.	0.16	0.50
C.M.	0.06	1.30
C.A.R.	1.85	3.10
C.A.	0.60	11.85
C.M.	1.00	2.60
D.A.G.	0.07	0.15
D.V.M.	2.26	0.35
D.A.	0.70	0.00
D.B.A.	1.59	0.30
D.C.A.	9.49	11.6
D.V.L.	2.80	3.00
D.F.	0.54	3.60
F.M.	8.87	9.60
L.A.	0.04	1.55
L.V.	0.86	0.55
L.V.	0.44	0.15
M.D.	4.63	3.30
M.C.	0.73	1.75
M.G.	2.32	2.80
M.A.	3.17	0.85
M.D.P.	0.35	0.05
M.B.	0.34	0.25
M.D.	0.33	1.15
M.R.	0.48	2.75
P.A.	0.11	1.10
P.T.	4.53	0.30
P.A.	29.2	7.80
P.R.	1.13	0.95
P.G.	8.00	21.0
R.A.	3.08	1.50
R.G.	0.49	0.75
R.M.L.	0.11	0.60
S.V.	3.50	7.30
S.G.	1.67	3.35
S.N.	1.29	1.85
S.R.	1.22	3.40
T.G.	1.85	0.90
V.V.	0.07	1.25
V.D.C.	3.96	4.15
V.R.	0.19	0.35
Average IgE title	**2.9**	**3.4**
Standard deviation	**4.8**	**4.5**

Tab. 14. 1: Fennel-specific IgE values in sera from 44 fennel allergy patients as determined by the automated ImmunoCAP Thermo Fisher assay. The IgE title was expressed as kU/l and values > 0.1 kU/l were considered RAST-positive. 2: Results of sera from 44 patients with fennel allergy analyzed by the RAST-capture assay developed in our laboratory.

3.5.2. Development of an in-house RAST-capture assay and analysis of the fennel allergy patients

A RAST-capture *in vitro* assay was developed in our laboratory as a flexible and inexpensive tool in order to carry out further investigations. To achieve this goal, biotinylation of the semi-purified fennel extract was first required. Therefore 2 ml of fennel extract (3.5 mg/ml) were subjected to gel filtration, through a Sephadex G 25 column and 20 fractions of about 500 µl were collected in order to obtain the proteins of the extract dissolved in the desired buffer (PBS w/o Ca^{++} e Mg^{++} 1 mM EDTA).

As it is known, gel filtration is a technique that allows not only separation of molecules, such as peptides, proteins, nucleic acids, etc., on the basis of their size, but also pH shift of a given sample. In this case, the pH of the semi-purified extract was 5, whereas biotinylation had to be carried out at pH=7. The 20 fractions collected, after determination of their protein content by the Bradford method, were divided into two pools: a pool including fractions from 10 to 20 (about 6 ml) and a pool including fractions from 6 to 9 (about 2 ml). (Fractions from 1 to 5 were discarded).

Pool 10-20 was concentrated using 3 Centricon ultrafiltration devices with a cut-off of 10,000 Da, generating a retentate with proteins > 10,000 Da and an ultrafiltrate with molecules < 10,000 Da. The protein content of each of them was determined by the colorimetric Bradford method. Retentate protein concentration was 4.6 mg/ml (2.07 mg in a volume of 450 µl), while protein content of the ultrafiltrate was below the sensitivity threshold of the method. This result suggests that, probably, the low molecular weight protein of 9 kDa, reported in literature and identified as a lipid-transfer protein (LTP), was not present. In order to confirm this finding, the fennel ultrafiltrate (possibly containing proteins < 10,000 Da) was concentrated by using 8 Microcon ultrafiltration devices (500 µl were loaded in each Microcon) with a cut-off of 3,000 Da That led to obtain a retentate with molecules > 3,000 Da and an ultrafiltrate with molecules < 3,000 Da. Their protein content was determined in both retentate and ultrafiltrate fractions. However, protein concentrations in both of them was

undetectable. This result would seem to confirm the absence of the 9 kDa protein in the semi-purified fennel extract, generated in our laboratory.

Pool 6-9 with a protein content of 5.2 mg, was biotinylated using the biotin disulphide N-hydroxysuccinimide ester. We used 1.8 mg of biotin disulphide N-hydroxysuccinimide ester, in proportion to the mass of the proteins to be biotinylated, according to the following formula: mg of reagent = mg protein to be biotinylated x 20 x 341.39/20,000, where 20 is the molar excess of the reagent (between 10 and 30), 341.39 is the biotin molecular weight and 20,000 is the estimated average molecular weight of the protein to be biotinylated. Biotin disulphide N-hydroxysuccinimide ester was dissolved in dimethylformamide (DMF) (1.8 mg in 100 µl) and an appropriate volume of this solution (72 µl) was added to pool 6-9.

In order to remove the unconjugated biotin reagent, the sample was once more subjected to gel filtration on a Sephadex G25 column. Twenty-three fractions 500 µl were collected and their protein content was determined by the Bradford method. The majority of the biotinylated fennel proteins was present in fractions from 6 to 9, which were pooled in a sample with a protein concentration of 1.9 mg/ml.

This biotinylated fennel extract was then used in the RAST-capture assay. The assay employed, in part, also components from a commercial kit, marketed by RADIM.

Briefly, an ELISA 96-wells microplate, with monoclonal anti-IgE antibodies coated on the surface of the wells, was used (Fig. 10.).

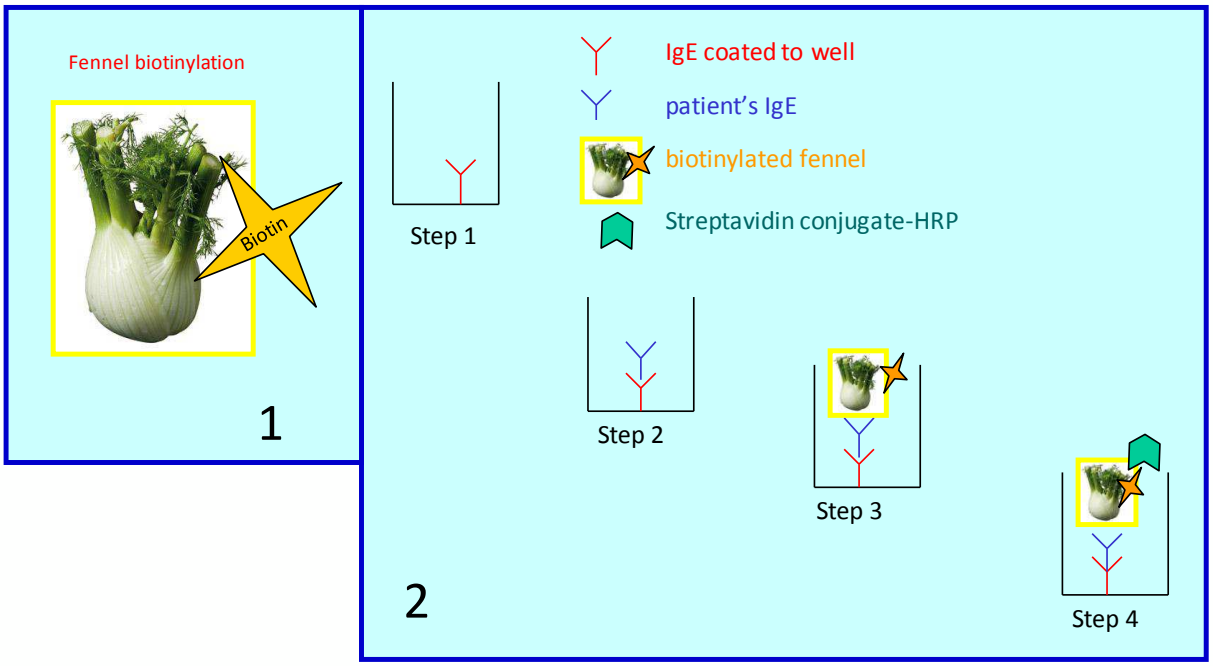

Fig. 10. Depiction of the ELISA capture *in vitro* assay developed in laboratory.

The patient's serum samples were added to the respective wells and the microplate was incubated at for 60' ± 5 at 37 °C. Thus, the IgE of the sample, both allergen-specific and non-allergen-specific were captured by the monoclonal antibodies, coated on the microplate wells. After extensive washing, 1 µg of the fennel allergen-biotin conjugated was added to the wells, previously incubated with the samples. Upon further incubation for 30' ± 5 at 37 °C, the biotinylated allergen bound the fennel-specific IgE, which, in turn, had been captured during the first incubation by the monoclonal anti-IgE antibody. The resulting immunocomplexes were made of: 1) a monoclonal anti-IgE antibody; 2) fennel-specific IgE and 3) biotinylated fennel proteins. After a second washing, streptavidin-peroxidase conjugated was added to the wells, allowing streptavidin to react with biotin. Finally, the addition of the peroxidase chromogen-substrate (a stabilized mixture of 3.3',5.5'-tetramethylbenzidine and hydrogen peroxide) revealed the fennel-specific IgE. The colour developed was proportional to the fennel-specific IgE title of the samples, which was determined by interpolation with the calibration curve. Two preliminary experiments were needed in order to find the appropriate amount of biotin-conjugated fennel proteins to be used in the assay (1 µg).

By this technique the 44 sera were analyzed. The results, expressed as kU/l, are also reported in Tab. 14.

Sera obtained from 14 non-allergic subjects were also analyzed by the in-house RAST-capture assay, as negative controls. The results of this analysis are shown in Tab. 15.

Patient	IgE Title (kU/l)
V.L.	0.30
M.D.G.	0.50
M.D.	0.20
A.N.	0.40
M.P.R.	0.15
I.F.	0.10
L.G.	0.40
R.R.	0.40
A.D.	0.15
D.D.N.	0.10
M.A.	0.20
L.M.	0.20
F.F.	0.00
L.M.	0.20
Average IgE title	**0.24**
Standard deviation	**0.14**

Tab. 15. Fennel-specific IgE values in sera from 14 non-allergic subjects (used as negative controls), as analyzed by the in-house RAST-capture assay. IgE values are expressed in kU/l.

Based on these results, a positivity threshold value (*i.e.*, the value above which the IgE titles obtained with the in-house RAST-capture assay could be considered positive) was calculated as being equal to 0.52 kU/l (average IgE title measured in negative controls plus 2 x standard deviation = 0.24 + 2 x 0.14 = 0.52 kU/l).

Therefore, by using this threshold value, 32 out of 44 fennel allergy patients analyzed (73%) were considered positive. Thus, 73% could be considered the sensitivity of this test, by using a 0.52 kU/l threshold value.

Average IgE value obtained in the 14 negative controls (0.24 ± 0.14 kU/l) was compared with the average value found in the 44 patients with fennel allergy (3.4 ± 4.5 kU/l), by the Mann-Whitney Rank Sum Test; a statistically significant difference between the two groups was found, ($p < 0.001$). See Fig. 11. This indicated that the RAST-capture assay developed in our

laboratory was specific and that the biotinylated fennel extract concentration of 1 μg/well adopted was appropriate.

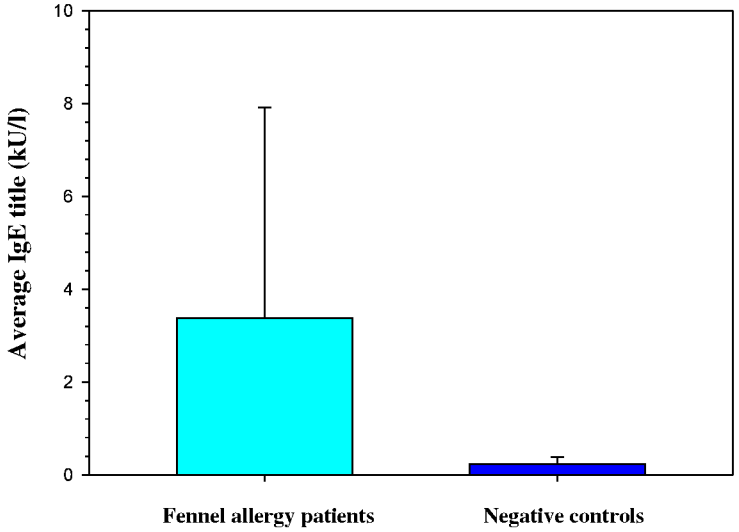

Fig. 11. Average IgE value, expressed in kU/l, in 44 fennel allergy patients *vs* 14 negative controls. Both groups were analyzed by the in-house RAST-capture assay.

3.5.3. Comparison between the two *in vitro* experimental approaches: ImmunoCAP Thermo Fisher assay and in-house RAST-capture assay

Detection of *F. vulgare* specific IgE was performed in the 44 sera obtained from fennel allergy patients by two different *in vitro* experimental approaches: the automated ImmunoCAP Thermo Fisher and the *in vitro* RAST-capture assay developed in our laboratory.

When the average fennel-specific IgE value in 44 patients studied using the RAST-capture assay (3.4 ± 4.5 kU/l) was compared with that obtained in the same patients with the ImmunoCAP Thermo Fisher (2.9 ± 4.8 kU/l) and subjected to statistical analysis, the Mann-Whitney Rank Sum Test revealed that the average IgE title obtained with the in-house assay did not significantly differ from that obtained using ImmunoCAP RAST ($p = 0.507$). See Fig. 12.

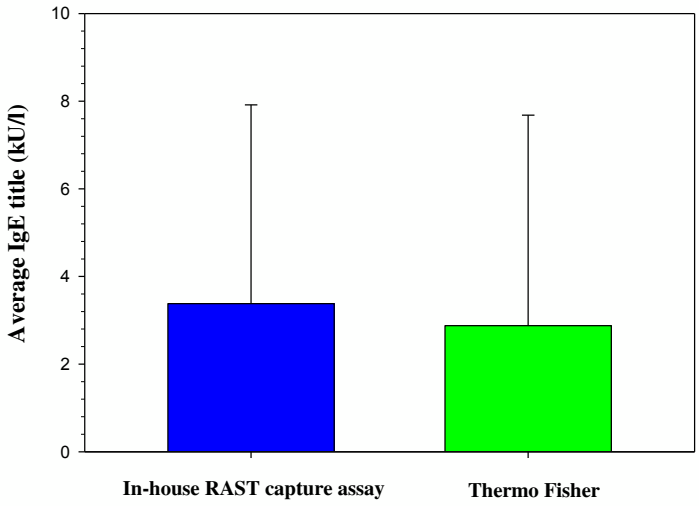

Fig. 12. Comparison between average IgE title (kU/l) obtained with the in-house RAST-capture assay and that obtained using the ImmunoCAP, marketed by Thermo Fisher (n=44 patients).

These data were also subjected to regression analysis. Thus, a linear correlation (linear regression) coefficient r = 0.518 was calculated (Fig. 13.). These findings suggested that there is a correlation between these two data populations. Therefore, the two experimental approaches could be considered comparable with each other, with a considerable degree of overlapping.

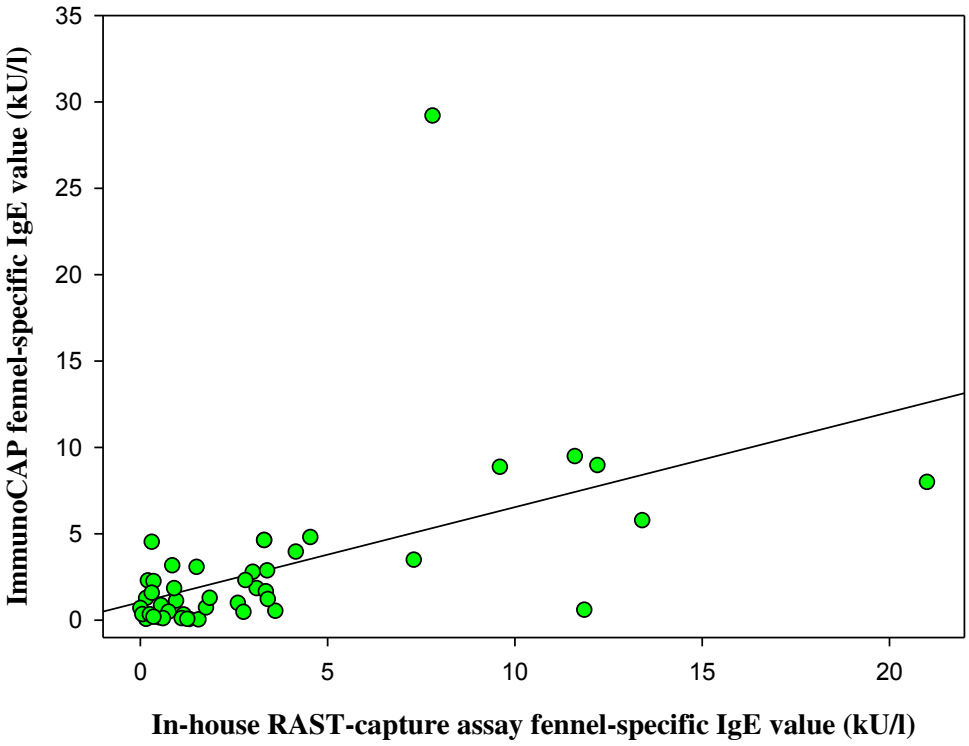

Fig. 13. Correlation between IgE fennel-specific values (kU/l) obtained by the RAST-capture assay and by ImmunoCAP assay (Pearson's linear correlation coefficient r = 0.518).

3.5.4. Finding the biotinylated fennel extract amount suitable for the in-house RAST-capture assay

In order to detect the appropriate amount of biotinylated fennel extract to be used in the in-house RAST-capture assay, several experiments were performed. First we selected 4 patients (B.L., F.M., P.A. and P.G.) out of the 44 patients studied, with relation to soundness of their clinical history, high skin reactivity to fennel extract and high fennel-specific serum IgE. We also selected 4 non-allergic subjects as negative controls. In a first experiment, 10 µg, 25 µg and 50 µg of biotinylated *F. vulgare* extract were tested. The results obtained indicated that these quantities were not satisfactory, since high levels of apparently specific IgE were measured in the sera of all the negative controls.

For this reason, sera from the same 4 fennel allergy patients as well as the sera from the 4 non-allergic subjects were analyzed using lower amounts of biotinylated fennel extract: 0.1 µg, 0.5 µg and 2 µg, respectively. This experiment revealed that 0.1 µg of biotinylated extract was probably insufficient, since IgE measurements in the sera of the fennel allergy patients

were too low (false negatives). On the other hand, 2 µg of biotinylated fennel extract could be considered too appropriate, too, since the 4 controls became slightly positive (false positives). Thus, considering these results, we adopted 1 µg of biotinylated fennel extract as the more appropriate amount of allergen to be used in the assay.

3.6. RAST inhibition experiments

The in-house RAST-capture assay, which implied the use of the biotinylated *F. vulgare* extract, was employed to carry out crucial RAST inhibition experiments. As it is known, this kind of experiments allow evaluation of the possible cross-reactivity between two allergenic proteins, which are supposed or suspected to share epitopes with each other.

Thus, if we consider an allergenic protein A, against which specific anti-A IgE have been produced and a second protein B, with potential common epitopes with protein A, protein B will also be recognized by the anti-A IgE. Therefore, RAST inhibition represents an easy approach for exploring this possibility.

In our setting, a serum known to contains IgE against protein A is pre-incubated for 24 h at 4 °C in the presence of increasing amounts of protein B. Successively, the sample is centrifuged at 20,000 x g for 30' at 4 °C (a pellet will precipitate, made of anti-A IgE protein B immunocomplexes, if present, since protein B will occupy the anti-A IgE binding sites). If we then measure the anti-A IgE title in the supernatant, using the same RAST method as before, we will detect a reduction of the title. This result will suggest that the two proteins cross-react with each other, probably because of the presence of common epitopes (Fig. 14.).

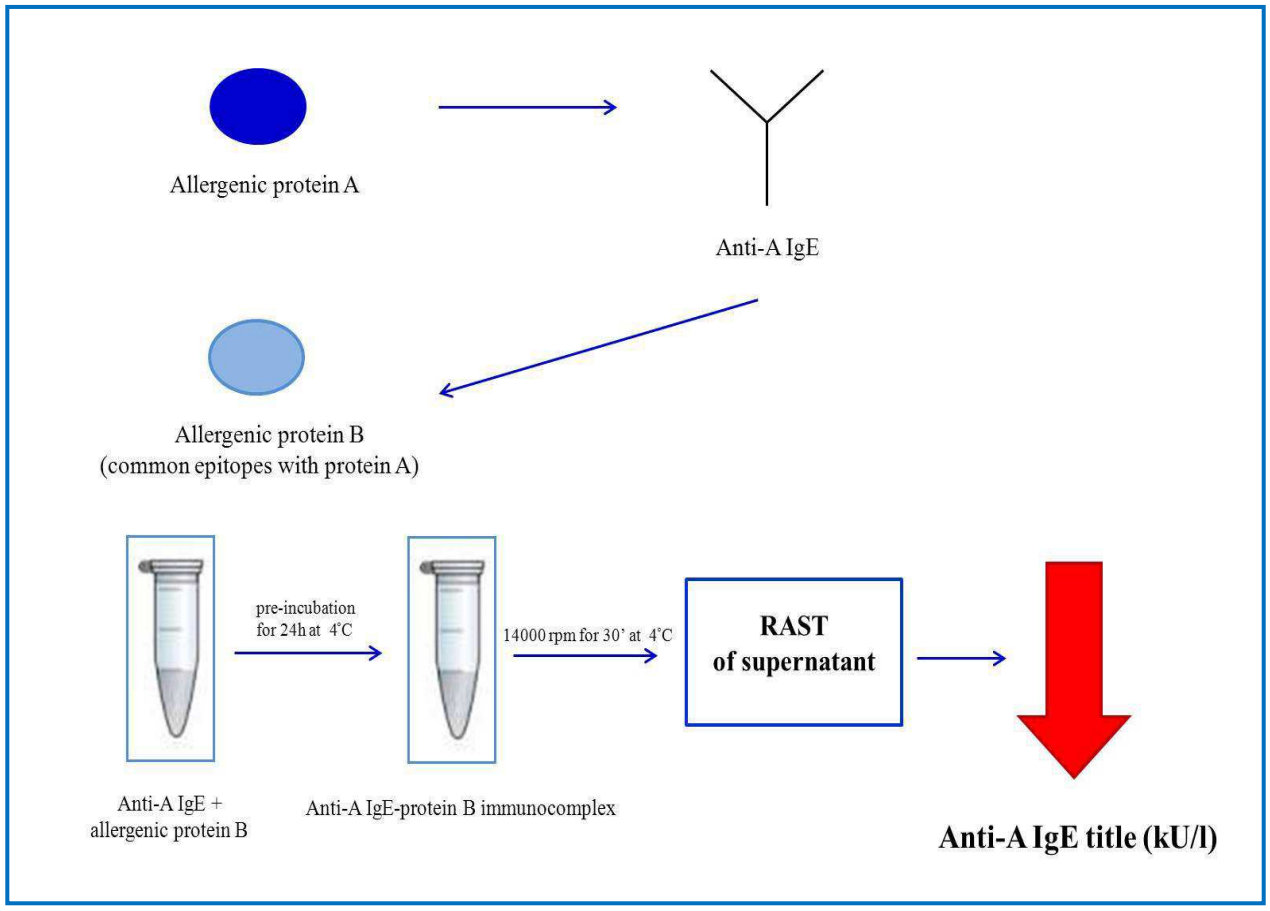

Fig. 14. Schematic depiction of RAST inhibition experiments, in our setting.

3.6.1. Production of the semi-purified extracts of peach and celery

Since cross-reactivity between fennel and peach had been suggested, we chose to use RAST inhibition as the experimental approach to evaluate the presence of cross-reactivity between these two Mediterranean plant foods. Moreover, since it seemed to us rather probable that cross-reactivity existed between fennel and celery (the former a novel food allergen, the latter a well-known one, both belonging to the *Apiaceae* family), we also decided to test this hypothesis by RAST inhibition.

To this purpose, peach and celery extracts from fresh food were generated in our laboratory. Suitable amounts (50 g) of the edible portion of fresh *P. persica* and fresh *A. graveolens* were homogenated, as described, and the homogenates were subjected to centrifugation (three times) and, successively, to ultracentrifugation, generating semi-purified 100,000 x g supernatants of the two plant foods. The protein content of the two extracts, determined by the colorimetric Bradford method, were 0.228 mg/ml for peach extract and 0.8 mg/ml for celery.

3.6.2. RAST inhibition experiments with the semi-purified peach extract

Four sera, chosen because of the high title of fennel-specifc IgE, according to ImmunoCAP Thermo Fisher results (patient B.L. = 8.97 kU/l; patient P.G. = 8.00 kU/l; patient C.C. = 5.78 kU/l; patient M.D. = 4.63 kU/l) were studied. As an example, the serum from patient B.L. was incubated for 24 h at 4 °C, on shaker, in the presence of increasing amounts of the semi-purified peach extract proteins: 0 µg, 0.228 µg, 1.14 µg and 4.5 µg, respectively. The 4 samples generated were successively centrifuged at 20,000 x g for 30' at 4 °C and 50 µl of the sample supernatant were analyzed by the in-house RAST-capture assay, described above. The results of this experiment, shown in Fig. 15., indicated that incubation of the serum with increasing amounts of peach proteins did not lead to a reduction of fennel IgE title, suggesting that fennel and peach do not cross-react. Similar results were obtained with the remaining 3 sera.

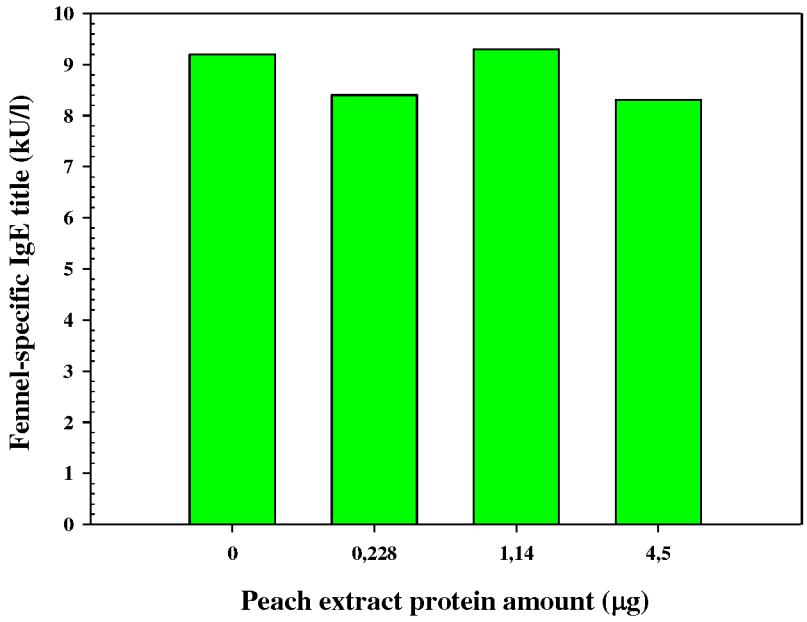

Fig. 15. RAST inhibition by peach. Patient B.L. serum was incubated with 0 µg, 0.228 µg, 1.14 µg and 4.5 µg of peach extract, respectively. No reduction in the fennel-specific IgE title was observed.

3.6.3. RAST inhibition experiments with the in-house *Foeniculum vulgare* extract

In front of the negative results of the RAST inhibition experiment carried out with the semi-purified peach extract, in order to assess the reliability and the experimental soundness of the approach adopted, we also performed RAST inhibition experiments with the in-house *F. vulgare* extract. In this case, a reduction of fennel-specific serum IgE title, upon incubation with the unconjugatedd fennel extract, would be expected, since biotinylated fennel extract proteins used in RAST would be recognized by the same antigen binding sites on specific IgE.

Therefore, the sera from the same 4 patients analyzed in the peach RAST inhibition experiments were studied.

Again, as an example, patient B.L. serum was incubated for 24 h at 4 °C, on shaker, in the presence of increasing amounts of the in-house fennel extract: 0 µg, 0.35 µg, 1.05 µg and 10.5 µg. The samples were then centrifuged at 20,000 x g for 30' at 4 °C and 50 µl of the supernatant of the pre-incubated samples were analyzed by the RAST-capture assay. The results obtained showed a gradual decrease of the fennel-specific IgE title (Fig. 16.), indicating that indeed incubation of the serum with unconjugated fennel proteins, dose-dependently removed fennel-specific IgE from the serum, causing the decline of the RAST title.

Again, completely similar results were obtained in the other 3 cases.

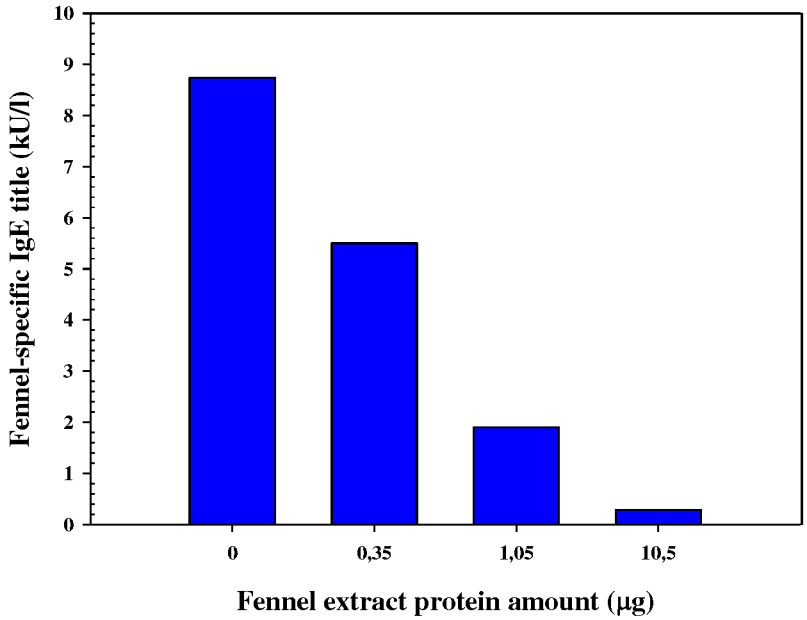

Fig. 16. RAST inhibition experiment performed with the in-house fennel extract. Patient B.L. serum was incubated with 0 µg, 0.35 µg, 1.05 µg and 10.5 µg of fennel extract, respectively, leading to a dose-dependent reduction of fennel-specific IgE title.

3.6.4. RAST inhibition experiments with celery (*Apium graveolens)* extract

Finally, RAST inhibition were also performed to evaluate the possible cross-reactivity between fennel and celery.

Celery (*A. graveolens)* is an edible vegetable belonging to the same taxonomic family of fennel and, therefore, phylogenetically near to *F. vulgare*.

Therefore, the sera from the same 4 patients as in the previous RAST inhibition experiments were incubated with increasing amounts of proteins contained in the in-house celery extract: 0 µg, 0.8 µg, 1.6 µg and 8 µg. These samples generated were kept for 24 h at 4 °C, while gently shaking and, successively, centrifuged at 20,000 x g for 30' at 4 °C. Fifty µl of the supernatant of the pre-incubated samples were analyzed by the RAST-capture assay for fennel-specific IgE title (see above). Again, as an example, in Fig. 17. the results relevant to patient C.C. are depicted. A dose-dependent reduction of the fennel-specific IgE title was observed also in this case. Thus, the reduction was proportional to the amount of celery proteins included in the samples. These results suggest that proteins of the semi-purified celery extract bound to fennel-specific IgE in the patients' sera, leading to the reduction of the IgE title, as measured by the RAST-capture assay.

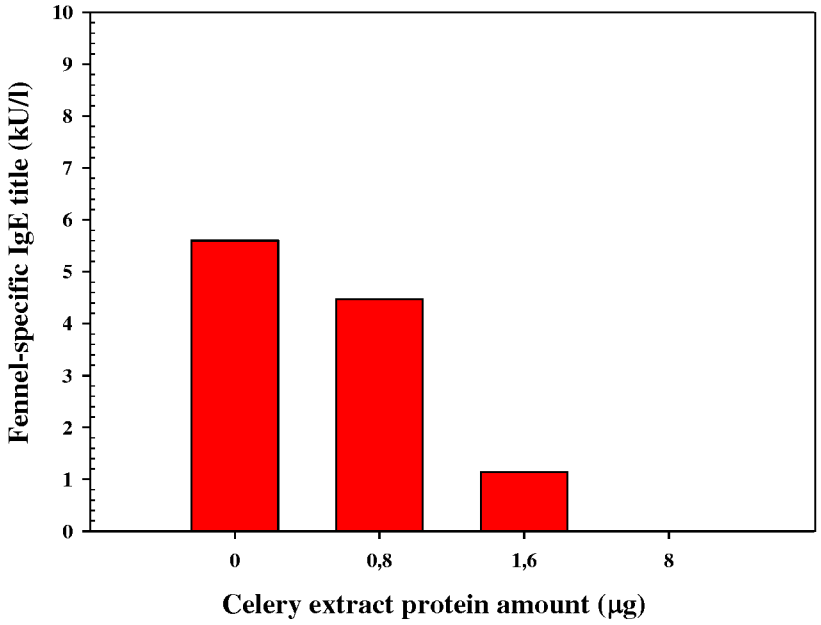

Fig. 17. RAST inhibition by celery 100,000 x g supernatant. Patient C.C. serum was incubated with 0 µg, 0.8 µg, 1.6 µg and 8 µg of celery extract proteins, respectively. A dose-dependent reduction of fennel-specific IgE title was observed, revealing the presence of structurally homologous proteins.

3.7. Immunoblotting analysis of the semi-purified *Foeniculum vulgare* extract

The semi-purified *F. vulgare* extract produced in our laboratory was used in Immunoblotting experiments to detect immunoreactive protein bands, possibly responsible for fennel allergy. Therefore, the in-house fennel extract was at first analyzed by SDS-PAGE. Experiments with resolving gel acrylamide concentration of either 10% or 12% (5% stacking gel) were performed in order to find out the optimal experimental conditions and to improve band resolution.

Moreover, from previous SDS-PAGE analysis under reducing conditions (see above), 45 µg of extract proteins was found to be the most convenient protein amount to be loaded in these experiments (as reported in the section 3.2.).

3.7.1. Experimental settings and technical experiments

In a first course of experiments, a 12% resolving gel acrylamide concentration was used in order to resolve protein bands with low molecular weight. In particular, our attention was focused on the putative 9 kDa band, described in literature by Others in similar experiments and recognized as a lipid-transfer protein, probably responsible for cross-reactivity between fennel and peach (Pastorello et al., 2013). Otherwise, the typical experiments were performed with a 10% acrylamide resolving gel.

In an early course of experiments, sera from the two most well characterized fennel allergic patients, P.A. and D.C.A., both with high levels of fennel-specific serum IgE (according to ImmunoCAP RAST results: 29.2 kU/l and 9.49 kU/l, respectively) were used for Immunoblotting analysis.

Several experimental conditions were tested in order to find out the optimal setting. In particular, we tested: a) various blocking conditions of the non-specific binding sites; b) primary antibody dilution (the primary antibody was represented by patient's serum); c) secondary antibody dilution (the secondary antibody was represented by either an anti-human IgE peroxidase-conjugated polyclonal antibody, raised in goat, or monoclonal anti-human IgE peroxidase-conjugated antibody); d) exposure time, after the ECL reaction step.

As for the blocking conditions, the whole nitrocellulose membrane was incubated in 50 mM TBS, pH 7.5, 5% non-fat dried milk for 1 h at room temperature. Alternatively, the blocking was carried out overnight at room temperature.

Successively, the nitrocellulose membrane was cut into strips, each corresponding to a single lane. The typical dimentions of the strips were 4 cm x 0.8 cm. The single strips were then incubated with the primary and the secondary antibody, respectively, and subjected to ECL reaction, in order to visualize the immunoreactive bands.

As for the primary antibody, dilutions of 1:100, 1:5, 1:2 were tried out. Typically, incubation of the strips with the primary antibody was carried out in 5 ml polypropylene tubes (Falcon, U.S.A.). The incubation buffer volume was 1 ml (50 mM TBS, pH 7.5, with the appropriate proportion of the patient serum, and 5% FCS, as the blocking agent). Other incubation conditions (e.g., open flat vessels with 5 ml incubation buffer) were occasionally tested. However, these alternative conditions were abandoned, due to limitation of primary antibody availability. In the typical setting, the incubation tubes were let to rotate at 11 rpm, either overnight at 4 °C or for 1 h at 37 °C. The 1:5 dilution / overnight / 4 °C setting was eventually selected as the most suitable.

Upon washing (see Materials and Methods section), the membrane strips were incubated with the secondary antibody. The following dilutions were experimented: 1:1000, 1:500, 1:400, 1:330 and, finally, 1:250. Incubation was carried out with the secondary antibody in 2 ml 50 mM TBS, pH 7.5, with the appropriate proportion of the secondary antibody, in the presence of 5% non-fat dried milk, as the blocking agent. Tubes as above were used. Incubation time was 3 h at room temperature, typically. Alternatively, experiments with incubation time of 1 h at 37 °C were performed. The final selected setting was: 2 ml / 3 h / at room temperature.

Intensity of immunoreactive band signal also depended on exposure time after ECL visualization. Thus, various exposure times were tested: 30'', 1', 3', 5', 15', 20', up to 1 h. The best results were obtained with a 15' exposure time.

Throughout the duration of the project, one of the most important emerged technical problem was represented by the strong background observed after film exposure. Thus, this part of the project was constantly developed and carried out with the purpose of finding out the right combination of these experimental conditions, in order to obtain immunoreactive bands as well-defined as possible. To this aim, we adopted the following strategies: increasing secondary antibody dilution and performing longer exposures; alternatively, decreasing secondary antibody dilution and setting up shorter film exposures.

At the end, the typical experimental conditions used in Immunoblotting experiments were: primary antibody diluted 1:5, first incubation overnight at 4 ºC; anti-human IgE peroxidase-conjugated polyclonal antibody diluted 1:330, second incubation for 3 h at room temperature; exposure time of 15'.

These conditions were used in analysis carried out with 40 out of 44 patients' sera available, since 4 sera with fennel-specific IgE values > 0.1 kU/l were discarded.

3.7.2. Artefactual nature of an apparently immunoreactive doublet

Analysis of the immunoreactive bands detected in the experiments carried out with the sera of 6 of the patients with the highest fennel-specific IgE values (P.A., D.C.A., F.M., P.G., C.C. and M.D.), revealed the presence of a protein doublet with high molecular weight (MW > 103 kDa; Fig. 18). Therefore, according to these results, a course of experiments was performed using a 10% resolving gel to better resolve higher molecular weight proteins.

Thus, a clear-cut immunoreactive doublet > 103 kDa was recognized also when individual sera from other 34 patients studied were employed in Immunoblotting. These experiments led us to believe that this doublet could be identified as the major allergen involved in *F. vulgare*

allergy. However, further Immunoblotting analysis performed with sera from 7 non-allergic patients, used as negative controls, showed an identical immunoreactive doublet (Fig. 19 A.), suggesting that the doublet might in fact be artifactual.

To confirm this hypothesis, other experiments were set up with fetal calf serum as the primary antibody (by definition, FCS does not contain antibodies). In these experiments, fetal calf serum was utilized as the primary antibody, diluted 1:5, while the same anti-human IgE peroxidase-conjugated polyclonal antibody of the previous experiments was used, diluted 1:330. The protein doublet with molecular weight > 103 kDa was also observed in this case, contradicting our early conclusions (Fig. 19 B.).

Therefore, for this reason, Immunoblotting experiments were repeated with an anti-human IgE peroxidase-conjugated monoclonal antibody (Abcam, Cambridge, UK). In these experiments, the presence of the protein doublet was not confirmed, therefore, adding to the assumption that the doublet recognition was probably artefactual, due to a non-specific binding between the anti-human IgE polyclonal antibody and proteins of the fennel extract.

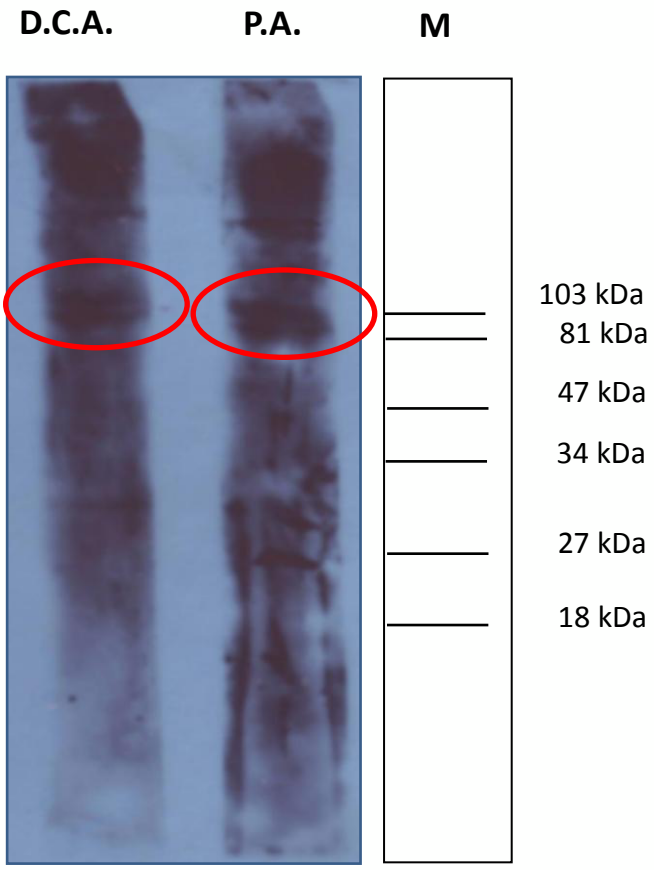

Fig. 18. Immunoblotting analysis carried out with sera from 2 different patients (D.C.A. and P.A.). An anti-human IgE peroxidase-conjugated polyclonal antibody (Sigma, Milan, Italy) was used as the secondary antibody. The protein doublet with high molecular weight (MW > 103 kDa) was visualized by ECL (exposure time 20').

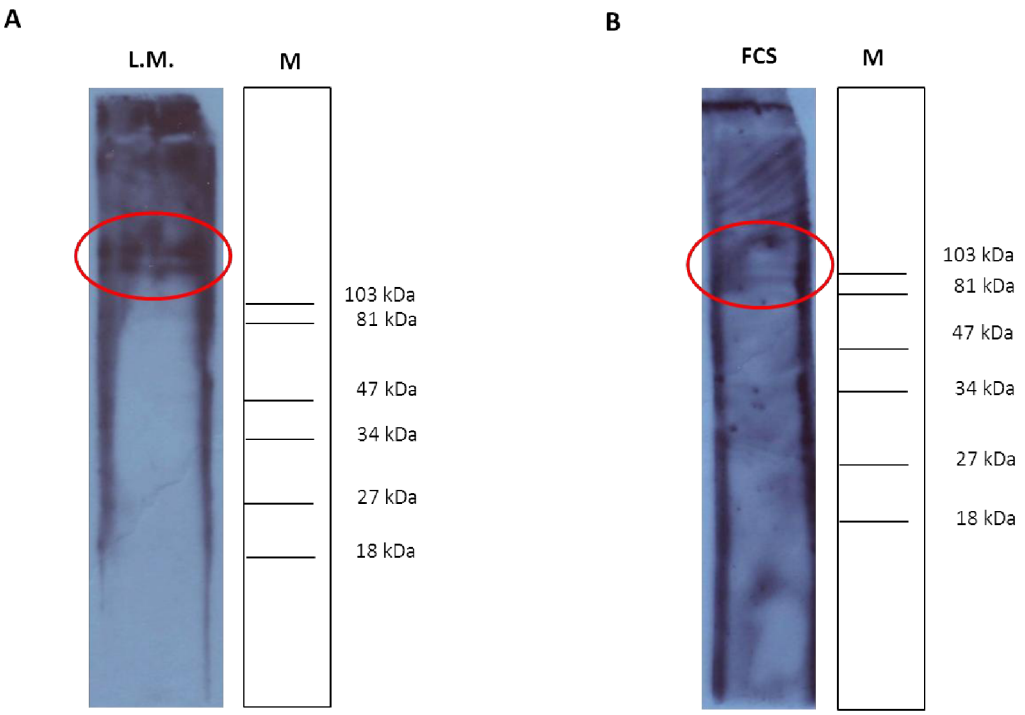

Fig. 19. Immunoblotting analysis carried out with: A) the serum from a negative control (L.M.) and B) fetal calf serum (FCS), used as the primary antibody, respectively. An anti-human IgE peroxidase-conjugated polyclonal antibody (Sigma, Milan, Italy) was used as the secondary antibody. The protein doublet with high molecular weight (MW > 103 kDa) was also observed in these cases (exposure time 15').

3.7.3. The sensitivity problem

An important as well as unavoidable factor of complexity and difficulty characterizing these experiments was the reduced sensitivity of this experimental approach due to the fact that the amount of specific IgE in the patients' sera, used as the primary antibody, was tiny if not negligeable. (In general, serum IgG are found in the order of magnitude of the g/l, whereas IgE are found in the order of µg/l, that is IgE are one million times less abundant than IgG). Therefore, to circumvent this technical problem, some experiments were repeated under modified experimental conditions: a) blocking of the non-specific binding sites was extended from 1 h to overnight, at room temperature; b) incubation time for the primary antibody and the secondary antibody was carried out at 37 °C (for 1 h).

However, these protocol modifications did not lead to a significant improvement of quality of immunoreactive bands resolution (weak signal intensity) or a reduction of background.

3.7.4. Detection of putative immunoreactive bands in 40 patients with fennel allergy

Immunoblotting analysis of the putative fennel allergens was performed with sera from 40 out of the 44 fennel allergy subjects studied.

Only patients with IgE values > 0.1 kU/l were considered. To detect immunoreactive bands, serum obtained from each patient (the primary antibody), diluted 1:5 overnight at 4 ºC and an anti-human IgE peroxidase-conjugated polyclonal antibody (as the secondary antibody), diluted 1:330 for 3 h at room temperature, were used.

The IgE-binding proteins were detected by ECL and, upon recordeing on RX films, the molecular weight of the immunoreactive bands was determined by comparison with markers with known molecular weight, loaded on the same gel. Prestained SDS-PAGE Standards – low range with the following molecular weight were used: 103, 81, 47, 34, 27, 18 kDa. In some experiments SDS-PAGE Precision Plus Protein Dual Color Standards were also used.

Besides the protein doublet with molecular weight > 103 kDa, the Immunoblotting analysis revealed rather diverse allergenic protein profiles, depending on the patient's serum used as the primary antibody.

Immunoblotting experiments were performed using sera from patients with fennel allergy, according to their fennel-specific IgE value, in descending order (Tab. 16.).

Patient	*% resolving gel*	*Secondary antibody dilution*	*Immunoreactive bands (kDa)*
P.A.	12 %	1:250	band with MW slightly < 81 kDa band with MW slightly > 47 kDa protein doublet in the 81-47 kDa range 47 kDa band 34 kDa band band in the 34-27 kDa range 27 kDa band band in the 27-18 kDa range 19 kDa band
D.C.A.	12 %	1:1000	protein doublet in the 81-47 kDa range 47 kDa band band in the 34-27 kDa range 27 kDa band band in the 27-18 kDa range 19 kDa band
B.L.	12 %	1:330	band with MW slightly < 75 kDa 50 kDa band 45 kDa band
F.M.	12 %	1:330	band with MW slightly < 75 kDa band with MW slightly > 47 kDa 50 kDa band 45 kDa band band in the 37-25 kDa range
P.G.	12 %	1:250	band with MW slightly < 75 kDa band with MW slightly > 47 kDa 50 kDa band 45 kDa band band in the 34-27 kDa range
C.C.	12 %	1:250	band with MW slightly < 81 kDa 47 kDa band 34 kDa band
M.D.	10 %	1:400	band with MW slightly > 47 kDa 18 kDa band
P.T.	10 %	1:400	band with MW slightly < 81 kDa band with MW slightly < 34 kDa
V.D.C.	10 %	1:400	band with MW slightly < 34 kDa
S.V.	10 %	1:400	band with MW slightly < 27 kDa 18 kDa band
M.A.	10 %	1:400	band in the 47-34 kDa range
R.A.	10 %	1:400	band with MW slightly > 47 kDa 27 kDa band

D.V.L.	10 %	1:500	27 kDa band
M.G.	10 %	1:500	34 kDa band
B.G.	10 %	1:500	band in the 34-27 kDa range 18 kDa band
D.V.M.	10 %	1:500	27 kDa band
C.A.R.	10 %	1:500	band in the 34-27 kDa range
T.G.	10 %	1:500	band in the 47-34 kDa range
S.G.	10 %	1:330	only the protein doublet with MW > 103 kDa was observed
D.B.A.	10 %	1:330	band with MW slightly > 18 kDa
S.N.	10 %	1:330	band in the 47-34 kDa range
C.A.	10 %	1:330	band in the 47-34 kDa range band with MW slightly > 18 kDa
S.R.	10 %	1:330	only the protein doublet with MW > 103 kDa was observed
P.R.	10 %	1:330	only the protein doublet with MW > 103 kDa was observed
C.M.	10 %	1:330	band in the 103-81 kDa range band with MW slightly > 34 kDa band in the 27-18 kDa range
L.V.	10 %	1:330	band in the 103-81 kDa range band in the 81-47 kDa range band with MW slightly > 34 kDa
M.C.	10 %	1:330	only the protein doublet with MW > 103 kDa was observed
D.A.	10 %	1:330	band in the 103-81 kDa range band with MW slightly < 47 kDa
C.A.	10 %	1:330	band with MW slightly > 34 kDa band in the 27-18 kDa range
D.F.	10 %	1:330	band in the 103-81 kDa range
R.G.	10 %	1:330	band in the 47-34 kDa range band with MW slightly < 18 kDa
M.R.	10 %	1:330	only the protein doublet with MW > 103 kDa was observed
L.V.	10 %	1:330	band with MW slightly < 47 kDa
M.D.P.	10 %	1:330	band with MW slightly < 47 kDa
M.B.	10 %	1:330	34 kDa band
M.D.	10 %	1:330	band with MW slightly < 47 kDa
V.R.	10 %	1:330	protein doublet with MW of 18 kDa
C.R.	10 %	1:330	band in the 34-27 kDa range band with MW slightly < 27 kDa
P.A.	10 %	1:330	band with MW slightly < 47 kDa band in the 34-27 kDa range

R.M.L.	10 %	1:330	band with MW slightly < 47 kDa band in the 34-27 kDa range

Tab. 16. Immunoreactive bands, corresponding to putative fennel proteins involved in *F. vulgare* allergy, as revealed by Immunoblotting analysis performed with sera from 40 out of 44 fennel allergy patients. Resolving gel acrylamide concentration and dilution of the secondary antibody are indicated.

Besides immunoreactive bands analysis carried out with the individual sera, common protein patterns were detected by some of the sera of the patients studied. Therefore, putative allergenic proteins involved in *F. vulgare* allergy were detected.

In particular, a band with an apparent molecular mass of ~ 50 kDa was detected by sera from 5 patients (P.A., F.M., P.G., M.D. and R.A.; Fig. 20 A.). Moreover, another immunoreactive band with an apparent molecular weight of ~ 45 kDa was detected by sera from 5 fennel allergy patients (L.V., M.D.P., M.D., P.A. and R.M.L.). Finally, yet another immunoreactive band with the apparent mass of 33 kDa (comprised in the range between 34 and 27 kDa markers) was identified by 8 of the sera studied (P.A., D.C.A., P.G., B.G., C.A.R., C.R., P.A. and R.M.L.; Fig. 20 B.).

Notably, 2 out of the 8 patients' sera (P.A. and R.M.L.) that recognized the 33 kDa band, also reacted toward the approximately 45 kDa band. Whereas, 2 that recognized the 45 kDa band (P.A. and P.G.) also recognized the approximately 50 kDa band.

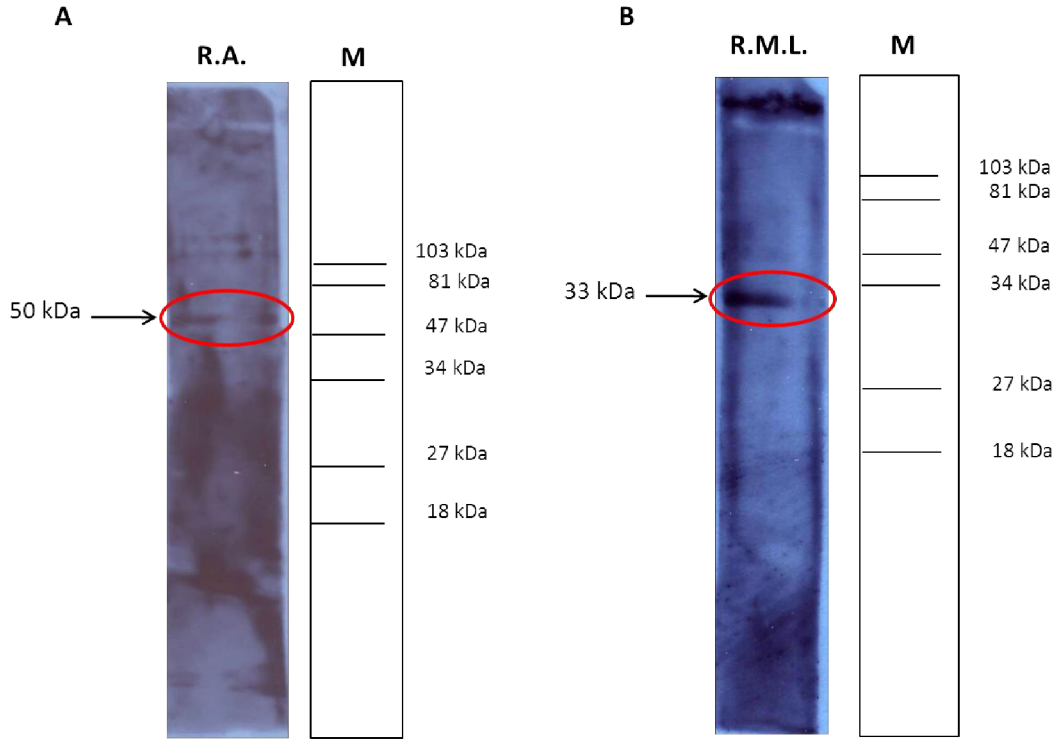

Fig. 20. A) The 50 kDa immunoreactive band detected by serum from the patient R.A.; B) the band with the apparent molecular weight of 33 kDa observed in the patient R.M.L. In both cases, an anti-human IgE peroxidase-conjugated polyclonal antibody (Sigma, Milan, Italy) was used as the secondary antibody. These bands were visualized by ECL (exposure time 10' and 15', respectively).

Moreover, two distinct patterns of bands were recognized by multiple sera, accounted for in Tab. 17.

Patient	*Immunoreactive band pattern (kDa)*
P.A. **D.C.A.**	protein doublet in the 81-47 kDa range 47 kDa band band in the 34-27 kDa range 27 kDa band band in the 27-18 kDa range 19 kDa band
B.L. **F.M.** **P.G.**	band with MW slightly < 75 kDa 50 kDa band 45 kDa band

Tab. 17. Two immunoreactive band pattern were detected by fennel allergy patients' sera. The first included 6 bands and was recognized by 2 patients sera; instead, the second included 3 bands and was recognized by 3 patients sera.

Finally, out of 18 distinct Immunoblotting experiments no evidence was found of immunoreactive bands with a molecular weight around or slightly below 10 kDa.

4. Discussion and Conclusion

Food allergy represents the focus of considerable research interest, as epitomized by the inclusion of this topic in the list of the thematic areas of the Sixth and Seventh Framework Programmes of the European Union.

Within this scientific and cultural framework, particularly important appears the characterization of novel food allergens, from a biochemical and immunological point of view and the appreciation of the exact magnitude of food allergy in any given homogenous geographical area. The latter goal is particularly important achieving, since food allergy is regarded as an increasingly common disorder in Europe and in other developed Countries.

Demand for safe food is on the rise as well concern for inexpensive continuous supply of our tables. Thus, food security is now considered an important element for the achievement and maintenance of public health. This is also true for those Countries bordering the Mediterranean basin, which benefit from the advantages of the peculiar combination of food and life-styles, commonly reffered to as the Mediterranean Diet.

In recent years, the Mediterranean Diet has attracted substantial interest, because of its benefit conferred on human health and its hypoallergenic properties. Thus, in general, foods belonging to the Mediterranean Diet less frequently cause allergy, but this Diet also includes potentially allergenic foods, most of which are poorly characterized or, simply, unrecognized.

An example, is represented by *F. vulgare*, a novel food allergen of the Mediterranean Diet belonging to the *Apiaceae* family, which has been seldom studied in the past.

First of all, this research project was aimed at defining the occurrence of fennel allergy, in a population with a typically Mediterranean Diet, from Apulia Southern Italy and characterizing *F. vulgare* proteins involved in this type of food allergy.

A population of 189 essentially adult patients with food allergy diagnosis, observed at our outpatient clinic, was screened for fennel allergy.

Among them, *F. vulgare* allergy was diagnosed in 57 patients (30%), who reported symptoms clearly associated with fennel consumption and exhibited positive SPT with fennel extract, consistent with the diagnosis of fennel allergy.

According to these results, it seems that fennel can be considered a major food allergen in those Countries, like Italy, where the Mediterranean Diet prevails. In particular, in a population from Apulia, Southern Italy, this type of food allergy accounts for a substantial proportion of all food allergy cases, possibly, up to 30%.

These 57 patients were, successively, invited to participate in a focused study, aimed at characterizing fennel allergy in immunological details. The study protocol included: a thorough revision of the clinical history; *in vivo* tests, consisting in SPT, performed with a commercial extract marketed by Lofarma and with an in-house semi-purified fennel extract, generated in our laboratory, respectively. Moreover, prick by prick tests were carried out with raw and microwaved fennel, respectively, and a blood sample was collected.

The patiens'sera were analyzed using ImmunoCAP Thermo Fisher RAST and a RAST-capture assay developed in our laboratory, upon fennel extract biotinylation.

Among these 57 fennel-allergy patients, 44 complied with the study protocol.

As for diagnosis, usually, SPT were carried out with an array of 34 commercial food allergens. Since commercial extracts must be considered as a mixture of several different proteins and proteins (from plant foods, in this case) could be denatured during the industrial preparation, a semi-purified fennel extract was generated in our laboratory.

This fennel extract was produced starting from the edible part of fresh fennel. However, its preparation posed many problems that were solved as follows: a) elimination of the insoluble fraction of the fresh fennel homogenate through various steps of centrifugation and an exhaustive ultracentrifugation at 100,000 x g; b) neutralization of any protease, in order to avoid the denaturation of fennel extrtact proteins using a vegetable protease inhibitor cocktail; c) neutral pH assignment to the fennel extract, achieved with the inclusion of PBS 10X w/o Ca^{++} e Mg^{++} in the sample to homogenize; d) finally, protein content determination, obtained by the Bradford method. By combining these different methodologies we generated an in-house semi-purified fennel extract with a protein concentration of 3.5 mg/ml.

This extract was produced in order to carry out the various *in vivo* and *in vitro* experiments, respectively, planned.

As regards the *in vivo* tests, the results obtained (Tab. 10) suggested that the average area of the skin prick test wheals obtained using the in-house *F. vulgare* extract (59 ± 47 mm^2) was approximately double than the average area of those obtained with the commercial extract, marketed by Lofarma, (26 ± 18 mm^2). That means that the sensitivity of the in-house extract was approximately 10 times higher compared to the commercial extract, given by the logarithmic relationship between the magnitude of the wheal elicited and the measured amount of mast cell-bound specific IgE.

Moreover, the average area of the prick by prick wheals (mm^2) obtained with raw fennel (42 ± 43 mm^2) did not significantly differ from that obtained with microwaved fennel (36 ± 32 mm^2; p=0.562), suggesting that the allergenic proteins involved are probably thermostable.

Moreover, it is worth noticing that the average area of the wheals elicited by the in-house semi-purified fennel extract was almost twice as big as compared to that of the prick by prick wheals obtained with fresh fennel (39 ± 40 mm^2), indicating that generation of the extract leads to a substantial concentration of the allergenic proteins. Once more, as it is known, the magnitude of wheal response reflects, according to a logarithmic relation, the amount of endogenous histamine released by activated cutaneous mast cells that, in turn, depends on the amount of the existing specific IgE linked to mast cell surface by the high affinity receptor FcεRI.

Therefore, on the basis of these considerations, it can be said that the in-house semi-purified *F. vulgare* extract seemed to elicite a greater wheal response than that of the fresh food and the commercial fennel extract.

The in-house fennel extract was also used for *in vitro* testing, as described above. Thus, upon biotinylation of the extract proteins, an in-house RAST-capture assay was developed, in order to detect *F. vulgare* specific IgE in the 44 sera from the fennel allergy patients recuited in our study. This was also done in order to have at hand a more flexible and inexpensive experimental tool.

Thus, upon biotinylation, followed by gel filtration, it was possible to obtain a reagent, with a protein concentration of 1.9 mg/ml, which was used in the in-house RAST-capture assay.

Analysis of the sera obtained from the 44 patients revealed that 32 out of the 44 fennel allergy patients analyzed (73%) could be considered positive, using a biotinylated *F. vulgare* extract concentration of 1 μg/well and considering 0.52 kU/l as the positivity threshold value for the test. This value was calculated from the average IgE title measured in 14 negative controls plus 2 x standard deviation (0.24 + 2 x 0.14 = 0.52 kU/l).

The average value calculated in the 44 fennel allergy patients was 3.4 ± 4.5 kU/l. The Mann-Whitney Rank Sum Test revealed that the difference observed between this average value and the value obtained obtained in the 14 negative controls (0.24 ± 0.14 kU/l) was statistically significant.

Therefore, these results indicated that the RAST-capture assay developed in our laboratory was sensitive and specific. On the other hand, the biotinylated fennel extract concentration of 1 μg/well adopted in the test was appropriate, based on the technical experiments described

above and the final outcome of the assay. In conclusion, this *in vitro* assay was reliable, easy to perform and characterized by acceptable sensitivity (73%) and good specificity (100%).

Moreover, the results obtained by the automated ImmunoCAP assay and the in-house RAST-capture assay developed in our laboratory were compared with each other, revealing that no major differences could be detected between these two different experimental approaches, as indicated by the Mann-Whitney Rank Sum Test. The average fennel-specific IgE value by RAST-capture assay in the 44 patients (3.4 ± 4.5 kU/l) proved not to be statistically different from the value obtained with the ImmunoCAP assay (2.9 ± 4.8 kU/l). This conclusion was corroborated by the correlation analysis performed with the Pearson's linear correlation Test.

However, although these two *in vitro* approaches were found comparable with each other as the results obtained demonstrated, the development of the RAST-capture assay offered several advantages. Thus, it allowed to study the immunological response to the in-house fennel extract proteins by measuring specific the IgE title (kU/l) at reduced costs, since the Thermo Fisher assay can be performed only on dedicated platform, available only at the level of major hospital routine laboratory. Moreover, the almost unlimited availability of the in-house *F. vulgare* biotinylated extract allowed repeatability of the experiments.

Thus, the in-house RAST-capture assay was employed to perform RAST inhibition experiments in order to evaluate the possible cross-reactivity between fennel and peach, which had been supposed to share some epitopes, inspite of the fact that peach belongs to the *Rosaceae* family and fennel to the *Apiaceae* family.

To the contrary, since fennel and celery belong to the *Apiaceae* family, it seemed to us rather probable that cross-reactivity would exist between these two edible vegetables.

Therefore, we carried out the appropriate RAST inhibition experiments in order to test the above hypotheses.

In fact, according to literature, a clear-cut cross-reactivity between fennel and peach was detected (Pastorello et al., 2013). These Authors performed Immunoblotting inhibition experiments, using a pool of sera from fennel allergy patients. These experiments revealed that pre-incubation of the sera with peach extract, at different dilutions, completely inhibited IgE binding to fennel proteins, suggesting a high cross-reactivity between the two foods.

Outcomes of our RAST inhibition experiments, carried out using the semi-purified peach extract, showed that fennel allergy patients' sera incubation, in the presence of increasing amounts of peach extract proteins (0 µg, 0.228 µg, 1.14 µg and 4.5 µg, respectively) did not lead to a reduction of the fennel-specific IgE title, therefore, suggesting that fennel and peach proteins do not cross-react with each other. These results were consistent with our clinical

data, according to which *F. vulgare* allergy can be regarded as a dominant and self-consistent food allergy.

Moreover, in front of the negative results obtained with the RAST inhibition experiments carried out with the semi-purified peach extract, RAST inhibition experiments with the same, non biotinylated in-house *F. vulgare* extract were performed, in order to ascertain the reliability of the peach/fennel RAST inhibition experiments. In this case, a reduction of fennel-specific IgE title, was found, as expected, since the unconjugated and the biotinylated fennel extract proteins compete for the same antigen binding sites at the hypervariable region of specific IgE.

The same outcome was observed in the RAST inhibition experiments carried out using the semi-purified celery extract, as hypothesized. Thus, pre-incubation of the sera from fennel allergy patients with increasing amounts of celery extract proteins (0 µg, 0.8 µg, 1.6 µg and 8 µg, respectively) revealed a gradual reduction in fennel-specific IgE title, indicating the existence of cross-reactivity between these two vegetables, as expected. Plausibly, this could be due to proteins characterized by a high homology degree that cross-react with each other.

Another important focus of this research project was the biochemical characterization of the proteins responsible for fennel allergy. To achieve this goal, the semi-purified fennel extract was subjected to SDS-PAGE and Immunoblotting analysis.

Thus, 40 out of 44 sera from fennel allergy patients with an IgE value > 0.1 kU/l (considered positive according to the ImmunoCAP Thermo Fisher RAST results) were analyzed. The results obtained, first of all, revealed much diverse allergenic protein profiles, depending on the different patients studied. However, 3 major immunoreactive bands, putatively involved in fennel allergy, were detected. These bands were: a 33 kDa band (detected by sera from 8 patients), a 45 kDa band (detected by 5 patients) and, finally, a 50 kDa band (also detected by 5 patients).

These results differ from those reported in literature and already cited (Pastorello et al., 2013). In particular, our experiments did not show the presence of the 9 kDa band, identified and recognized, by Pastorello and co-workers, as a lipid-transfer protein (LTP). This band, according to these Authors, should be putatively involved in the cross-reactivity between fennel and peach.

This could be due to the different experimental protocols used in the fennel extract generation and also to the different sensitivity of the Immunoblotting techniques adopted. Thus, in the study cited above, fennel extract proteins were separated in a discontinuous gel with a 6% stacking gel and a 7.5-20% separation gel and the specific IgE-binding proteins were detected

by incubation with an ^{125}I-labeled anti-human IgE antibody. Instead, the Immunoblotting experiments performed in our laboratory were carried out using a continuous gel with a 5% stacking gel and a 10% (or 12%) separation gel and the immunoreactive bands were revealed by an anti-human IgE peroxidase-conjugated polyclonal antibody and ECL visualization.

Notably, in carrying out our experiments we were faced with background and aspecificity problems, after film exposure. A significant example is represented by the protein doublet with a high molecular weight (MW > 103 kDa), initially regarded, wrongly, as a major allergen of *F. vulgare*. In fact, this protein doublet was observed not only in fennel allergy patients, but also in non-allergic subjects. Moreover, experiments using FCS (which, by definition does not contain antibodies) were carried out. Also in this case, the protein doublet with molecular weight > 103 kDa was observed. Therefore, Immunoblotting experiments were repeated with an anti-human IgE peroxidase-conjugated monoclonal antibody. These experiments did not reveal the presence of the protein doublet, suggesting that it was probably artefactual, due to a non-specific binding between the anti-human IgE polyclonal antibody and fennel extract proteins.

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