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Detection of casein-IgE interaction in cow's milk allergic children through impedenzimetric measurements

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Content

Abstract

Background: Cow's milk allergy (CMA) is one of the main food allergies in children. Currently, CMA is usually diagnosed based on clinical history and evidence of specific IgE (s-IgE) to cow's milk (CM). The s-IgE can be documented by skin prick test (SPT) or measurement of s-IgE in the serum. These diagnostic tools are not able to predict the severity of allergic reaction when the patient ingests the allergen. Despite the improved accuracy conferred by s-IgE to individual allergens (i.e casein), compared with IgE to allergen extracts (i.e CM), a significant proportion of patients who are assessed for possible CMA require oral food challenge (OFC), which is currently the reference standard to diagnose CMA. The OFC is a procedure not free from the risk of having significant allergic reactions, even life-threatening, such as anaphylaxis.

Objective: The aim of the present study is to provide an alternative and more efficient method for diagnosis of CMA by measuring the impedance values of a patient's serum sample before and after its contact with an allergen of interest, in this case CM-casein, through an impedance measurement device.

Methods: we enrolled children with a personal history of reaction to CM as well as patients without history of CMA (control group) referred to Allergy Unit of the Meyer Children's Hospital, Florence, Italy (from September 2019 to September 2020). Children underwent SPT to CM and CM casein and a blood sample were collected to determine serum s-IgE to CM and CM casein. Patients with positive SPT or detectable CM casein-IgE underwent to OFC with baked milk. The OFC clinical manifestations were classified as mild, moderate, or severe and consequently the CM allergic patients were classified in different severity degree, in particular highly allergic (HA), medium allergic (MA) and low allergic (LA) children. For the impedenzimetric measurement an impedance measurement device was used.

Results: Overall, 33 patients were enrolled: 15 children with CMA and 18 not-CM allergic children. The measurements obtained by impedance measure device showed difference, in term of IgE binding kinetic, between CM allergic children and not-CM allergic children. Moreover, it was possible to detect impedance variation between CM allergic group that can discriminate the grade of severity of CMA.

Conclusions: Therefore, this new proposed approach improves the performance of the current test for serum s-IgE concentration in terms of assay time and, furthermore, provides information about the IgE binding kinetics that is related to the severity of the allergic reactions. Our results, although promising, need to be confirmed by more experiments.

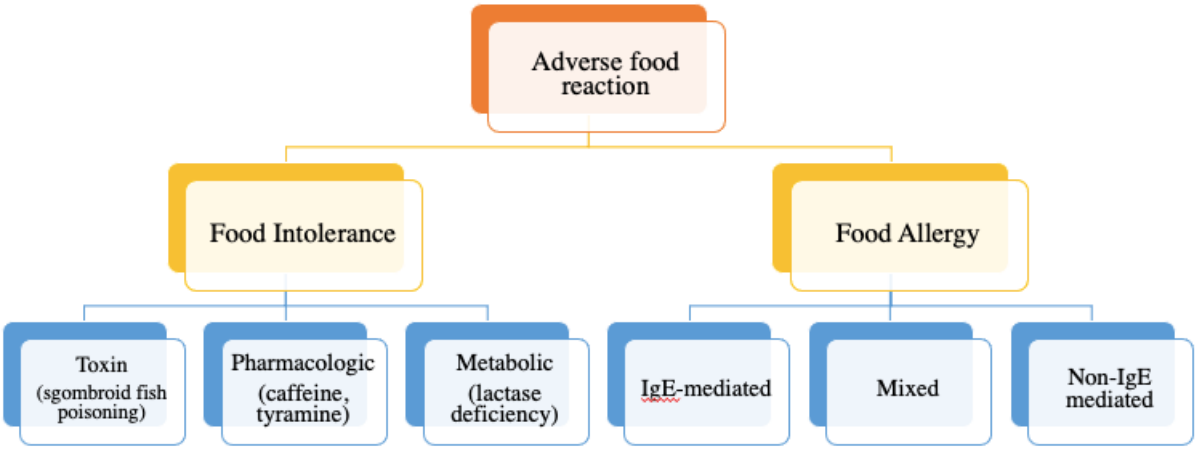
1. Background

1.1 Definition of food allergy and classification

Food allergy (FA) is an adverse reaction to a specific food antigen, normally harmless to the healthy population, which is mediated by immunological mechanisms and arises in a susceptible individual [1][2]. FA, therefore, differs from the so-called food intolerances, which exhibit the same symptoms but recognize different pathogenetic mechanisms. Intolerances are defined as non-immune reactions, mediated by toxic, pharmacological, metabolic, and undefined mechanisms. Milk intolerance due to the deficiency of the enzyme lactase, normally present in the brush border of the intestinal mucosa, and adverse reactions to foods characterized by a high histamine content or histamine-liberating substances, such as strawberries, chocolate, alcoholic drinks, and fermented cheeses, are examples of food intolerances [3]. In the past, food allergies (FAs) and intolerances were often confused with each other, due to their clinical similarity. Moreover, the same food is often responsible for both intolerance and allergy, rendering diagnosis difficult.

What distinguishes FA from other adverse reactions to foods is, therefore, the underlying pathogenetic mechanism: FA is an adverse reaction arising from a specific immune response that occurs reproducibly on exposure to a certain food. Moreover, based on the specific immunopathogenic mechanism, it is possible to distinguish in: a) Immunoglobulin E (IgE) - mediated FA which are mediated by antibodies belonging to the IgE and are the best-characterized food allergy reactions; b) non-IgE mediated or cell-mediated FA when the cell component of the immune system is responsible of the food allergy and mostly involve the gastrointestinal tract; c) mixed: IgE-mediated and non-IgE mediated when both IgE and immune cells are involved in the reaction [4]. The classification of adverse reaction to foods is shown in Figure 1. This thesis will focus on the IgE-mediated FA.

Figure 1. Classification of adverse reactions to foods (modified from [5][6]).



1.2 Epidemiology

The prevalence of FAs has increased in the last two to three decades and represents a public health problem, especially in industrialized countries [7]. The exact prevalence of FAs in a population is difficult to determine; in fact, the gold standard for diagnosis is the double-blind placebo-controlled food challenge (DBPCFC), which is not free of risk for the patient, and can only be performed in specialized centers [7].

For this reason, most of the studies are based on self-reported evidence or parent reports. It is clear, and already known, that this has led to an overestimation of FA prevalence. In fact, very often, the surveys are based on questionnaires that do not differentiate between IgE- and non-IgE-mediated FAs but are based on reported symptoms that are not confirmed by a DBPCFC [8][9].

FA mainly affects children [10] but an increasing number of elderly subjects also have symptoms of FA [11]. Age-related gender di differences are reported in FA [12]. For a long time, FA has been considered an almost exclusively pediatric disease because in the majority of cases it begins in childhood and tends to disappear with growth. However, the current exponential growth of the adult and elderly population, especially in Western countries, and the environmental and lifestyle changes, have profoundly changed the epidemiology of FA with a growing increase even in advanced age. Moreover, FA in aging exhibits peculiar clinical features and immunopathogenic mechanisms, increasing the diagnostic complexity [13].

The exponential growth of FA and the significant variability of the geographical distribution are the result of the influence of environmental factors and lifestyles on genetic

predisposition [14]. The highest prevalence of FA has been observed in Australia, reaching 10%of the infant population [15][16] whereas in Europe [17] and in the US [18], between 6% and 8% of children suffer from FAs, respectively.

FA includes a wide spectrum of clinical manifestations, from mild forms with organ localization, to serious and potentially fatal forms with systemic involvement. Almost half of patients with IgE-dependent FA have experienced at least one serious anaphylactic reaction, especially in childhood and adolescence [10][19]. The variability of clinical expressions and the complexity of the underlying immunological mechanisms contribute to making diagnosis often difficult and complicate the studies on the epidemiology of FA [7] [13][20][21][22][23][24][25][26][27][28].

Although each type of food may constitute a potential allergen, the list of foods responsible for the great majority of cases, especially the most clinically severe forms, is relatively short [3][23][29]. In industrialized countries the foods most frequently responsible for allergies in children are cow's milk, eggs, wheat, fish and shellfish, peanuts, walnuts, and soybeans [30]. Fish and seafood, peanuts and nuts, and fruit and vegetables are the prevalent causal allergens in adults. Furthermore, in relation to the eating habits, different foods may also be responsible for allergic sensitization in other countries.

For example, school-age Mexican children more often exhibit hypersensitivity reactions to chocolate, strawberries, crustaceans, and eggs [31], while sensitization to nuts and peanuts is more common in Chile [32], to chili, walnuts, chocolate, milk, and prawns in El Salvador [33], and to fruits, vegetables, and seafood in Colombia [34][35].

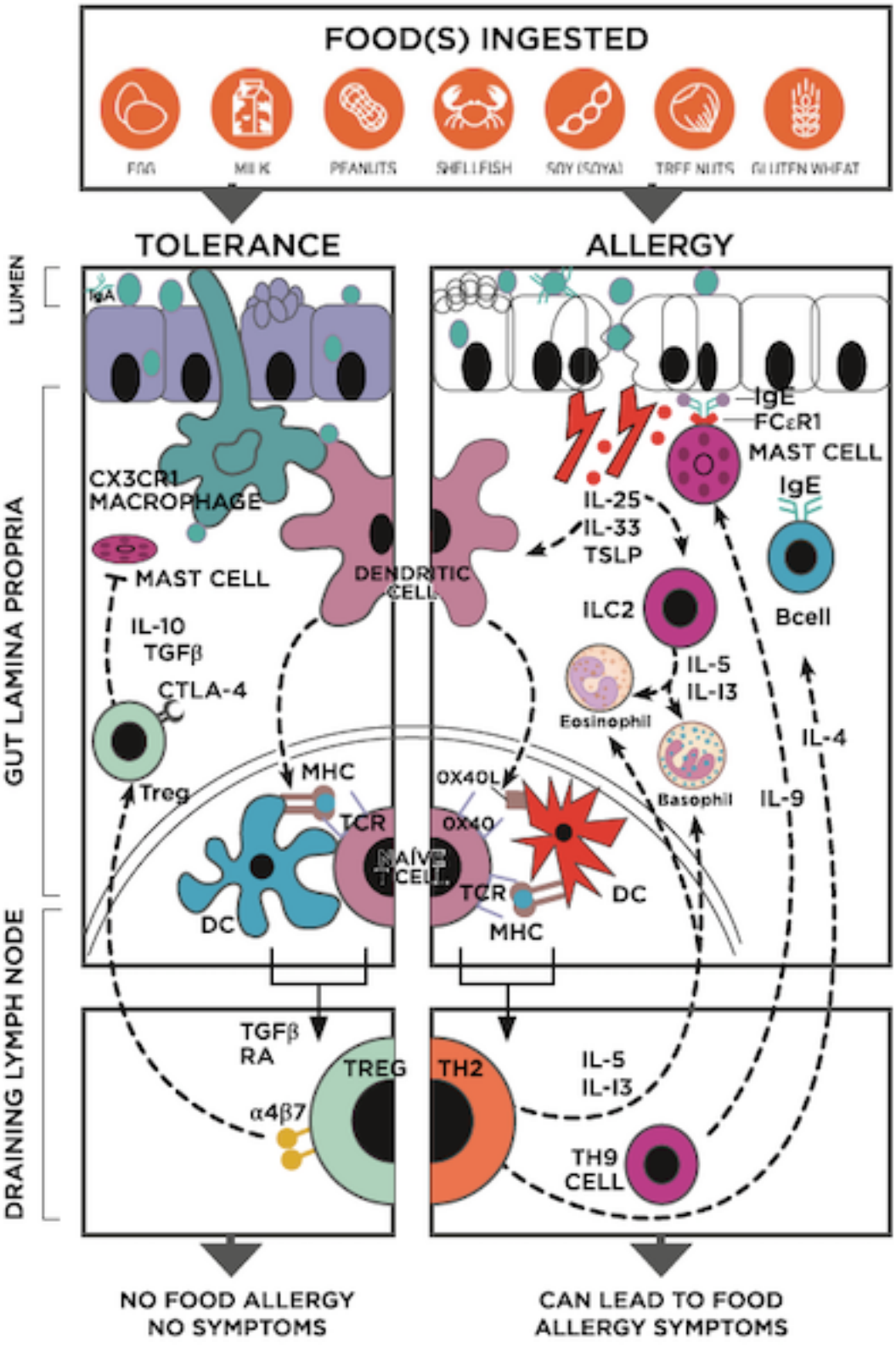
1.3 Pathogenesis

The breakdown of immunologic and clinical tolerance to an ingested food is the trigger for FA [3]. IgE-mediated FA is characterized by immediate clinical manifestations, due to the release of mediators triggered by the bonding of IgE antibodies, mast cells and basophils [7]. Oral tolerance consists in the systemic suppression of cellular and humoral immune response to an antigen first encountered in the gastrointestinal (GI) tract [36], although immune tolerance can be induced by other routes such as airways and intact skin [37]. Physical barriers, digestive processes, specific immune cells, and immune modulation determine gastrointestinal tract's ability to develop oral tolerance. Specialized GI cells (microfold, intestinal epithelial, and dendritic cells) play an essential role in antigen presentation and oral tolerance development, process food proteins outliving the digestive process. Dendritic

cells (DCs) play a central role in induction and maintenance of tolerance to food antigens. After antigen uptake they migrate into the mesenteric lymph nodes where they determine activation and differentiation of effector T cells. DCs determine active generation of food-antigen-specific regulatory cells (Tregs), which are probably influenced by the local microbiome [38][39]. Treg cells determine a regulatory, tolerant immune response by the production of transforming factor beta (TGF-β) and inhibitory cytokines (IL-10) through a retinoic acid-dependent mechanism [40]. The subsequent actions of both T and B cells are suppressed by TGF-beta and the latter also aids the production of secretory IgA [41]; T-cell anergy is induced by IL-10 which also sustains Treg populations. The switching of B-cell class to produce secretory IgA is also partially provided by IL-10 [42]. Sensitization is a condition of having detectable food antigen specific IgE which can precede (or sometimes follows if clinical tolerance develops) the development of clinical FA. Immunologic mechanisms leading to the sensitization start with the first contact when the allergen occurs. Disruption of food tolerance is a consequence of epithelial barrier damage following the exposure to many factors such as pathogen-associated molecular patterns (PAMPs). In response to injury, epithelial cells produce pro-inflammatory cytokines such as IL-25, IL-23 and thymic stromal lymphopoietin (TSLP) and DCs activation [43]. This induces danger signals, inflammatory cytokines release and dendritic cells activation. The activated dendritic cells in turn activate naive T cells into acquiring a T helper cells 2 (Th2) phenotypes, which promotes inflammatory signals, inducing food Ag-specific B cells to class switch and produce food antigen-specific IgE. Skin antigen exposure has also been associated with sensitization. Factors breaking immune tolerance through the skin include skin barrier defects consequent to filaggrin's mutations, a protein essential for skin integrity [44][45], damage to the skin, microbial adjuvants such as staphylococcus enterotoxin B. All these factors induce an innate inflammatory skin response causing sensitization [37]. The respiratory route is also responsible for triggering sensitization: inhaled aeroallergens can cross-react with food antigens, resulting in an oral allergy syndrome. Once sensitization has been established, re-exposure to the antigen can led to local or systemic manifestations. Once produced, IgE bind to its high-affinity receptor FcεRI on the surface of mast cells and basophils, therefore arming these cells for activation on re-exposure to the antigen. The second contact with the antigen activates and makes these cells degranulate, resulting in performed mediators release (histamine, tryptase, platelet activating factor, prostaglandin and leukotrienes) and can lead to local and systemic manifestations [46]. The comparative

overview of the tolerogenic versus allergic response to food antigen in the gut is shown in Figure 2.

Figure 2. Comparative overview of the tolerogenic versus allergic response to food antigen in the gut (from [6]).



Legend:
Tolerance (left): food antigens are sampled from the intestinal lumen by CX3CR1 macrophages, which then transfer the antigen to dendritic cells in the gut lamina propria. Dendritic cells (DCs) can transfer processed antigen to the draining lymph nodes, and in the context of non-inflammatory mediators, such as transforming growth factor-beta (TFG-β) and retinoic acid, DCs present food peptide by way of the major histocompatibility complex (MHC) to the T cell receptors (TCR) on naïve T cells. This interaction promotes the differentiation of naïve T cells into food antigen-specific T regulatory cells (Tregs). Food specific Tregs then travel to the lamina propria via α4β7 where they encourage the maintenance of tolerance to food antigen via CTLA-4 expression and the release of cytokines TGF-β and IL-10. CTLA-4 inhibits Th2 T cells while TGF-β and IL-10 suppress the effector cells that promote allergy such as mast cells and also encourages the maintenance of IgA in the lumen.
Allergy (right): in the presence of gut epithelial damage, pro-inflammatory cytokines, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) are secreted and promote the expansion of type 2 innate lymphoid cells (ILCs) and activation of dendritic cells. Activated DCs, in the presence of these proinflammatory cytokines, will take up and process antigen to peptide and upregulate the expression of the surface protein OX40L. The peptide: major histocompatibility complex (MHC) on DCs and TCR on the naïve T cells interact, as well as the OX40L on DCs and OX40 on naïve T cells. This interaction promotes the differentiation of naïve T cells to type 2 helper T cells (Th2). Th2 cells and ILCs can secrete proinflammatory cytokines such IL-5 and IL-13, thereby promoting eosinophil and basophil recruitment in the gut lamina propria leading to downstream target effects that promote allergic sensitization. Th2 cells also secrete IL-4, which allows for B cell class switching to promote food specific IgE production. Th9 cells also play a major role in the development of the allergic response by secretion of IL-9, which promotes the recruitment of mast cells

Several hypotheses have been formulated to explain the increase of FA:

a) The hygiene hypothesis. A lack of microbes and infections exposure in early childhood might increase susceptibility to allergic disease by altering the development of the immune system through an imbalance of the immune responses in favor of the Th2 lymphocyte profile rather than Th1 [47]. Observational studies suggest that factors associated with increased microbial exposure, such as exposure to pets, childcare attendance, vaginal delivery and presence of older siblings, might have protective effects against developing food allergy [48][49][50]. The dual-allergen hypothesis. Several studies conducted in animals and humans suggest that disrupted skin barrier function in infant eczema might cause allergen sensitization through environmental exposure via the skin rather than oral route [51]. This hypothesis also supposed food antigens skin exposure is more likely to lead to allergy compared to early oral consumption, which is more likely to lead to tolerance [52].

FA is likely a combination of both skin and gut exposure to a food antigen, with a higher tendency towards sensitization if the first exposure is through the skin.

b) The Vitamin D hypothesis. Vitamin D has well recognized immunoregulatory and tolerogenic functions, and its deficiency is considered a possible risk factor for FA development [53]. Vitamin D was first related to the prevention of FA by demonstrating that infants with vitamin D level <50 nmol/L at 1 year of age had an 11-fold higher risk of peanut allergy, confirmed by oral food challenge, compared to infants with vitamin D levels >50 nmol/L [54]. Some evidence suggests that vitamin D is important in the regulation of Th cells differentiation and the induction of T-reg cells [55] and Th2 immune responses have been shown to be favored under low vitamin D or vitamin D-deficient conditions [56][57][58]. The Western diet along with fruit and vegetable low intake and low sun exposure may represent a risk of vitamin D deficiency, which could potentially enhance.

c) The microbiota hypothesis. The presence of specific bacterial strains as well as dietary substrate and their metabolites, could influence FA development [59] [60].

d) The "false alarm hypothesis". Smith et al. [61] very recently proposed a different theory to explain FA increase. The Western diet is high in advanced glycation end-products (AGEs) deriving from cooked meat, oil and cheese, and high concentration of sugar. They suggest AGEs, that are present or formed from the food in our diet and are alarmins, prime innate signaling, leading to development of FA [62].

1.4 Clinical presentation

FA has been nicknamed the great transformer. In fact, it is not a single disease, nor is it caused by a single pathophysiologic disturbance [63]. The type and severity of symptoms changes from one subject to another, and in the same subject from one reaction to another, in accordance with food and with the same food depending on the sensitizing molecule. Symptoms most frequently affect the skin, GI, respiratory and cardiovascular system, in either isolation or in association. The short period of time (usually < 2 hours) between ingesting a food and the appearance of symptoms leads to a suspicion of a mediated IgE reaction, except for food dependent exercise anaphylaxis (FDEIA) and of delayed anaphylaxis to red meat [64]. Cow's milk allergy (CMA), for example, can occur with several different immune mechanisms that induce rather different clinical frameworks. In IgE-mediated CMA, clinical history is often characteristic and repetitive. Symptoms often

arise within two hours of the first exposure to cow's milk and include cutaneous symptoms (such as flushing, urticaria, angioedema, pruritus) and/ or GI symptoms (such as nausea, regurgitation, vomiting, and sometimes diarrhea) and/or sometimes other symptoms such as crying or lethargy, etc. Clinical manifestations are extremely evident and in most cases regress within a few hours and reappear following further exposure to cow's milk protein [19]. Anaphylaxis is the most severe of allergic reactions. Anaphylaxis involves at least 2 body systems and is defined as "a serious, life-threatening generalized or systemic hypersensitivity reaction" and "a serious allergic reaction that is rapid in onset and might cause death" [65][66]. Patients with anaphylaxis commonly present symptoms involving skin or mucous membranes, followed by respiratory and gastrointestinal and cardiovascular symptoms.

1.5 Diagnosis

Diagnosing food allergy has major implications for daily life. It may lead to dietary restrictions and constant risk assessment to minimize accidental exposure and allergic reactions. This in turn may cause anxiety of varying severity and limit activities in which individuals may engage, such as attending social events, eating in restaurants, and travelling. [67][68]. Therefore, overdiagnosis can have an unnecessary negative impact on patients' lives. Conversely, failure to diagnose food allergy can place the patient at risk for reacting unexpectedly to food, with potential severe consequences. Thus, it is extremely important to get the diagnosis right. Currently, food allergy is usually diagnosed based on a history of an acute allergic reaction that happens within 2 hours (usually minutes) of allergen exposure [69] and evidence of allergen specific IgE (s-IgE) to the food in question. Allergen s-IgE can be documented by skin prick test (SPT) or measurement of s-IgE in the serum using allergen extracts or individual components, which can be either natural purified or recombinant proteins [70].

Despite the improved accuracy conferred by s-IgE to individual allergens for some foods, compared with IgE to allergen extracts, a significant proportion of patients who are assessed for possible food allergy require oral food challenge (OFC), which is currently the reference standard to diagnose food allergy [19][71]. In fact, offering allergy tests such as SPT and s-IgE testing ideally should come with access to OFC to minimize overdiagnosis of food allergy. OFC is the current reference standard for diagnosis, assessment of severity, identification of the threshold dose of reactivity, confirmation of eligibility for treatment,

and assessment of response to treatment [19][71]. However, oral food challenges (OFCs) have limitations, require intensive resources, and can cause allergic reactions of varying severity [72]. The need to undergo OFCs to confirm eligibility can leave patients and their families unsettled and anxious and may discourage allergic patients from considering specific treatments for food allergy. Therefore, improved biomarkers for food allergy are needed to close these gaps, provide an accurate diagnosis, and reduce the requirement for OFC.

1.5.1 Skin prick test

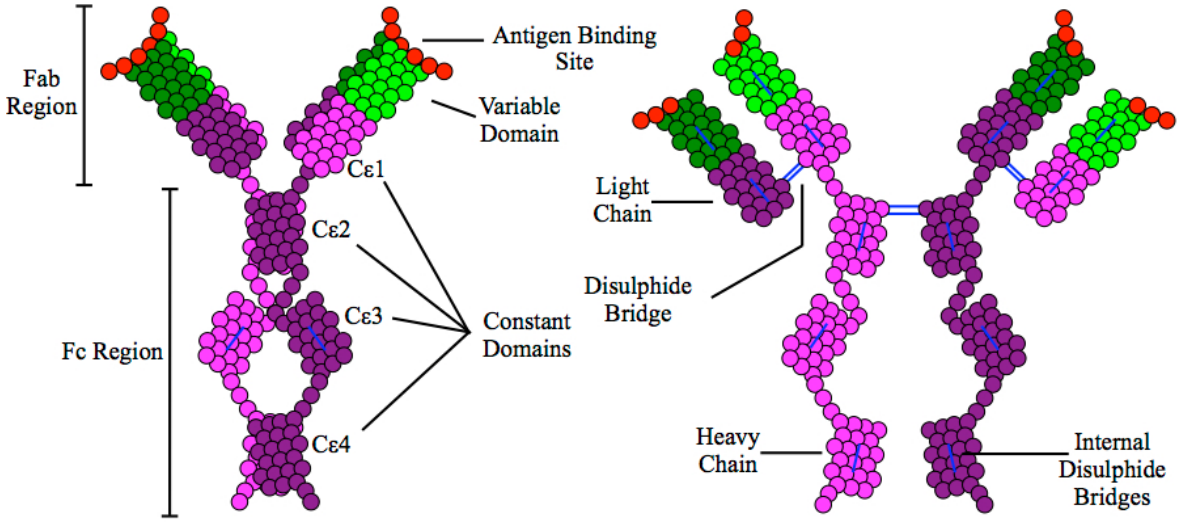
The SPT is a rapid method of screening for the presence of allergen s-IgE antibodies bound to cutaneous mast cells. The test is performed by using a device coated with commercial extract or fresh food and scratching the surface of the skin. This introduces the food protein through the skin, which can then bind to s-IgE on cutaneous mast cells and trigger degranulation of preformed mediators like histamine. The activation of the local mast cells produces a wheal and flare visualized at the site where the allergen was introduced through the skin. The wheal is measured 15 min after the prick and the diameter of the wheal provides information on the likelihood of clinical reactivity. A mean wheal diameter ≥ 3mm larger than the negative control is considered a positive skin test and could suggest the likelihood of clinical reactivity [73]. However, a positive SPT only reflects the presence of s-IgE antibodies bound to mast cells and is not diagnostic of clinical reactivity. The presence of s-IgE antibodies without clinical reactivity is called sensitization. Therefore, caution should be made when interpreting results and only suspected food allergens should be tested, since food skin testing has a low specificity [74][75].

A positive SPT cannot differentiate between sensitization and a true IgE-mediated allergy; therefore, it is important that the clinician combine a detailed clinical history and the results of a test to determine the need for additional diagnostic testing to support an IgE-mediated food allergy. Although wheal size does not correlate with disease severity, positive predictive values for clinical reactivity have been reported based on the size of the wheal diameter. For example, a mean wheal diameter size ≥8 mm for cow's milk, ≥7 mm for hen egg, and ≥8 mm for peanut was 95% predictive of reaction if challenged [76]. Skin-prick tests (SPTs) have a relatively high NPV and can be particularly useful in ruling out IgE-mediated food allergy to a specific food. However, clinical reactivity occasionally occurs with a negative SPT [4]. Intracutaneous testing, also called intradermal testing, for the diagnosis of food allergy is not recommended given the risk of serious adverse reactions [4]

1.5.2 Specific IgE (s-IgE)

Immunoglobulins are heterodimeric proteins composed of two heavy chains and two light chains. They can be functionally separated into variable domains that bind antigens and constant domains that specify effector functions, such as activation of complement or binding to Fc receptors. Each variable domain can be split into three regions of variability in the sequence called Complementarity-Determining Regions, and four regions having a relatively constant sequence called the framework regions. The binding between the antigen and the antibody's variable domain is weak and essentially non-covalent. Electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions are all known to be involved depending on the interaction sites [77]. The IgE is a very potent immunoglobulin, although it is present at the lowest serum concentration and has the shortest half-life. IgE is associated with hypersensitivity and allergic reactions, as well as with the response to parasitic worm infections. IgE binds with extremely high affinity to the receptor known as FcεRI, the high-affinity receptor for the Fc region of IgE, which is expressed on mast cells, basophils, Langerhans cells, and eosinophils. Circulating IgE upregulates the FcεR expression on these cells. The combination of strong binding and upregulation of FcεR expression contributes to the remarkable potency of this immunoglobulin. IgE was initially identified in 1967 as the reagin that mediates type I hypersensitivity [78].

Figure 3. IgE structure (from [79]).



a. Specific IgE to allergen extract

s-IgE testing has been used to diagnose FA for many years. IgE is quantified using kilounits per liter (kU/L) based on the World Health Organization Reference Standard with 1 unit

equaling 2.42 ng of IgE [80]. The measurement provides evidence of unbound circulating allergen s-IgE levels produced. Food s-IgE levels correlating with a 95% positive predictive value for several foods have been previously determined and can potentially correlate with a reaction to a given food. One hundred children (62% male; median age, 3.8 years; range, 0.4–14.3 years) were evaluated for food allergy and diagnosed by history or OFC. On the basis of the previously established 95% predictive decision points for egg, milk, peanut, and fish allergy, greater than 95% of food allergies diagnosed in this prospective study were correctly identified by quantifying serum food s-IgE concentrations [81][82].

s-IgE levels should be interpreted with caution when the history is not supportive of an IgE-mediated allergy, especially given the high false-positive rates. In cases where the history is strongly supportive of tolerance to a food, for example, if the patient has eaten a large amount of the food recently without symptoms, it is better to avoid testing specific IgE levels. Like food allergen skin testing, the sensitivity of s-IgE testing is greater than 90%; however, the specificity of both tests can be less than 50% when assessing foods, due to possible cross-reactivity between related proteins [4].

A positive sensitization test does not necessarily prove that the cause of a reaction is food and some children with positive sensitization for food do not clinically react when food is ingested, so an OFC is often needed, especially when the previous clinical history is not clear, to establish the diagnosis of food allergy before recommending its use or exclusion from the diet [19]. On the other hand, a positive sensitization test for a given food can confirm allergy, especially when combined with a clear recent history of reaction to the food [4]. A higher level of s-IgE is linked with a greater probability of a clinical reaction, but not with its greater severity [4]. A negative result to SPTs and allergen s-IgE test make an IgE-mediated form of food allergy very unlikely [19].

b. Specific IgE to allergen component (allergen-component resolved diagnostic testing-CRD)

Conventional IgE testing uses natural extracts containing a complex mixture of proteins. Allergen s-IgE to component allergen tests for IgE binding to single allergens, allowing more precise profiling of the allergen s-IgE repertoire. s-IgE testing to components is available for single allergens (Singleplex-ImmunoCap Phadia-Thermo-Scientific) and for multiple allergens in microarrays (Multiplex-ImmunoCap ISAC), testing a range over 100 purified allergens simultaneously. A significant number of allergenic molecules contained in food

have been characterized up to date, and their number is increasing [83][84]. A recent EAACI Molecular Allergology User's guide proposed that Molecular Diagnostics can improve total allergen IgE testing where: (a) there are low abundant and/or labile food proteins in conventional allergy tests, (b) provides indicators of food-related cross-reactivity or (c) markers of genuine (species-specific) sensitization, (d) provides information on risk or severity associated molecules [85].

An example of this latter aspect could be done using cow's milk as allergen. Casein (Bos d 8), beta-lactoglobulin (Bos d 5), and alpha-lactoglobulin (Bos d 4) are the major allergens in cow's milk. Sensitivity to various cow's milk proteins is widely distributed; thus, generally no single allergen is considered to be immunodominant [86]. In some studies, Bos d 8 was the best predictor of challenge-proven cow's milk allergy (CMA) [87][88]. In a Spanish study, the optimum cutoffs for Bos d 8 increased with age; using 2 kU/L (13-18 months), 4.2 kU/L (19-24 months), and 9 kU/L (24-36 months) gave a sensitivity of 95% and a specificity of 90% [89].This observation is important with regard to cutoffs for transient food allergies, such as cow's milk and egg, as one would expect that children who persist with CMA beyond 2 years would have higher casein levels than those who have already grown out of their CMA. In fact, IgE antibodies directed against sequential casein epitopes are a marker of persistent CMA [90]. High casein-IgE antibodies are predictive of baked CMA as casein is more resistant to extensive heating [91].

Other indications for the use of CRD include idiopathic anaphylaxis, delayed red meat anaphylaxis, wheat-dependent exercise-induced anaphylaxis, to differentiate between high versus low-risk molecules from foods giving rise to food-induced anaphylaxis (peanuts, nuts, shrimps, etc.), baked egg or milk allergy (ovomucoid, casein), etc. On the contrary, it is of little use when there is a convincing history of IgE-mediated allergy and a positive SPT or s-IgE to the relevant whole food allergen; this information is already enough to make a diagnosis [84]. However, multiplex assays have some limitations such as they reveal sensitization to molecules with potentially no clinical relevance as they are all tested independent of the patient's history [92][93].

c. Specific IgE to peptides

IgE specificity can be refined further by determining the allergen epitopes to which IgE binds. This has been evaluated using short linear allergen peptides of 15 to 20 amino acids bound to a solid phase (eg, microarray or spot membrane) using immunofluorescence. Beyer et al. [94] identified 5 immunodominant epitopes in selected peanut allergen peptides in

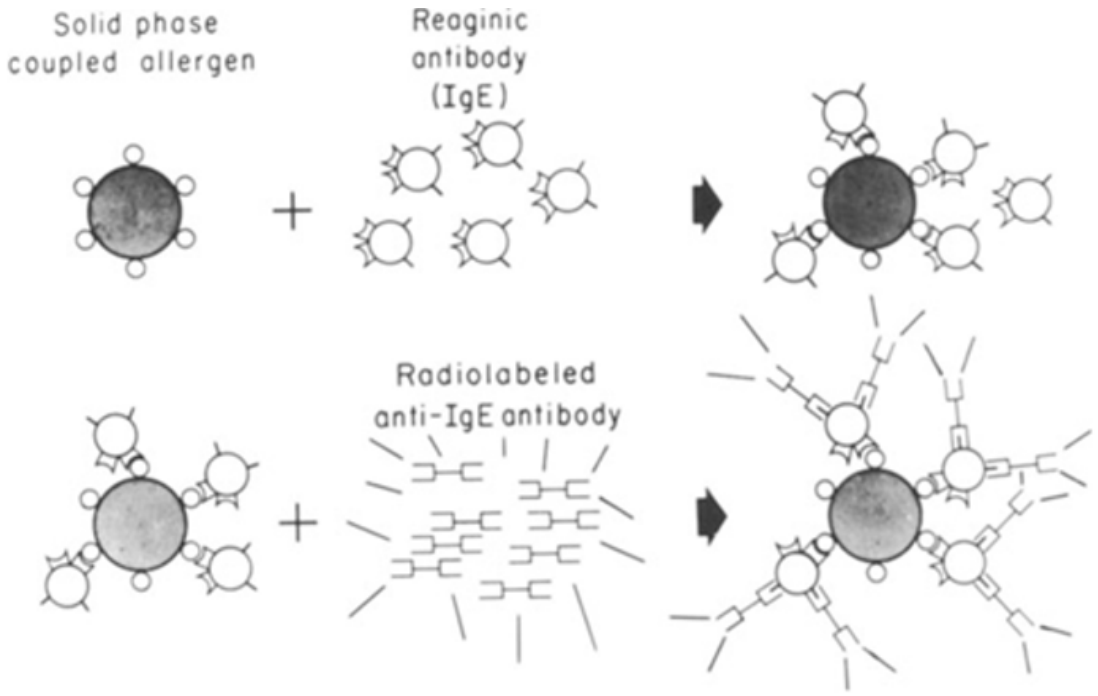
2003. Years later, a microarray containing peptides of the major peanut allergens, Ara h 1, Ara h 2, and Ara h 3, identified epitopes bound more by the IgE of peanut allergic patients than by the IgE of peanut sensitized-tolerant patients; this allowed the development of a machine-learning method that markedly enhanced the diagnostic utility of the microarray [95]. Similar methods have tested the utility of IgE to allergen peptides in diagnosing and in predicting the resolution of other food allergies [96][97][98][99][100]. In a CMA study, IgE binding was more diverse and had higher affinity for cow's milk allergen peptides in milk allergic patients reacting to baked milk compared with patients who reacted to unheated milk but tolerated baked milk, suggesting that the peptide microarray could be useful in identifying different phenotypes of CMA [96].

Although this technology appears to hold great promise, it requires knowledge of the amino acid sequences of component protein allergenic epitopes comprising each individual food, large cohorts of well-characterized food-allergic patients to validate the assays, and extensive computational skills and machine learning to develop and validate diagnostic and prognostic algorithms for each food allergen [101].

1.5.2.1 IgE assay methods

To date, several methods have been developed to detect serum specific IgE (s-IgE). In 1972 the first commercial assay for allergen specific IgE called radioallergosorbent test (RAST), was developed [102]. The original RAST test was configured as a test carried out on a paper allergosorbent disc to which many different allergens of different specificities were covalently coupled. Serum was incubated with this solid-phase allergen, permitting antibodies of all isotypes to bind thereto. After a buffer wash to remove unbound proteins, bound IgE antibody was detected with radioiodinated polyclonal antihuman IgE. After this second incubation and a second buffer wash, bound radioactivity was quantified in a gamma counter (Figure 4). The entire test required three days. The quantity of counts per minute bound was proportional to the amount of IgE antibody specifically bound to the immobilized allergen.

Figure 4. Diagrammatic representation of the radioallergosorbent test (RAST) (from [103]).



In more recent years, the improvements of technology in the solid-phase component have led to the development of the ImmunoCAP system (ThermoFisher Diagnostic) for in vitro testing [104]. This test uses a three-dimensional cellulose solid phase as a replacement of the two-dimensional paper disks of the modified RAST. As a result, the required incubation times are shorter. These newer IgE antibody assays use a larger number of allergen extracts, also having higher quality, for preparing the related allergosorbents. New matrix materials such as the cellulose sponge used in the ImmunoCAP System have enhanced the binding capacity, while reducing the nonspecific binding properties of allergosorbents. Various polyclonal and monoclonal anti-IgE detection antibody combinations insure maximal assay sensitivity and specificity for human IgE. Nonisotopic labels have increased the shelf life of reagents and have made the assays more user-friendly and safer to perform. Moreover, automation has improved assay precision and reproducibility to the level where some IgE antibody assays on auto-analyzers require only single measurements to insure accurate results. Calibration systems used in the second-generation IgE antibody assays have employed a common strategy in which a heterologous total serum IgE curve is used to convert allergen s-IgE assay response data into quantitative dose estimates of IgE antibody. The entire test takes about six hours for completion.

Two further diagnostic assays for the determination in laboratory of specific serum IgE have been developed. One is called Immulite® system (Siemens, Germany) and uses a

biotinylated allergen that is bound to an avidin solid phase. The other one is the HyTech-288 system (Hycor/Agilent Technologies, California, US) that uses a cellulose wafer on which the allergen is covalently coupled. The above mentioned three systems are all performed on auto-analysers to maximize precision and minimize the turnaround time. They all use nonisotypically labelled antihuman IgE and are calibrated by means of interpolation of response data from a heterologous total serum IgE calibration curve that has been referenced to the World Health Organization (WHO) total IgE serum standard (Analytical performance characteristics and clinical utility of immunological assays for human immunoglobulin E antibodies of defined allergen specificities" [105].

1.5.2.2 A new diagnostic tool: impedance meter

It has been recently suggested an alternative method to measure IgE-allergen interaction by means of impedenzimetric measurements [106]. The impedance is generally defined as the total opposition that a circuit, or more in general an object, offers to the flow of alternating current (AC) at a given frequency [107].

The idea at the basis of the proposed measurement technique relies on the fact that the interaction between the IgE and the corresponding allergen takes place through an electrochemical bonding mechanism which changes serum impedance according to the number of IgE which bind to the allergen [77] [108]. Following this interaction some of the free protons/electrons of the immunoglobulin structure come closer to the allergen structure, thus enhancing, or respectively reducing the impedance value of the serum under testing. In other words, the higher is the difference in the impedance values measured, the higher is the interaction between the IgE and the allergen under testing and therefore, the higher number of IgE bonding to the allergen. This new diagnostic tool, providing the IgE binding kinetics to a specific allergen, offers a meaningful information on the severity of allergic respond linked to the bonding speed [106].

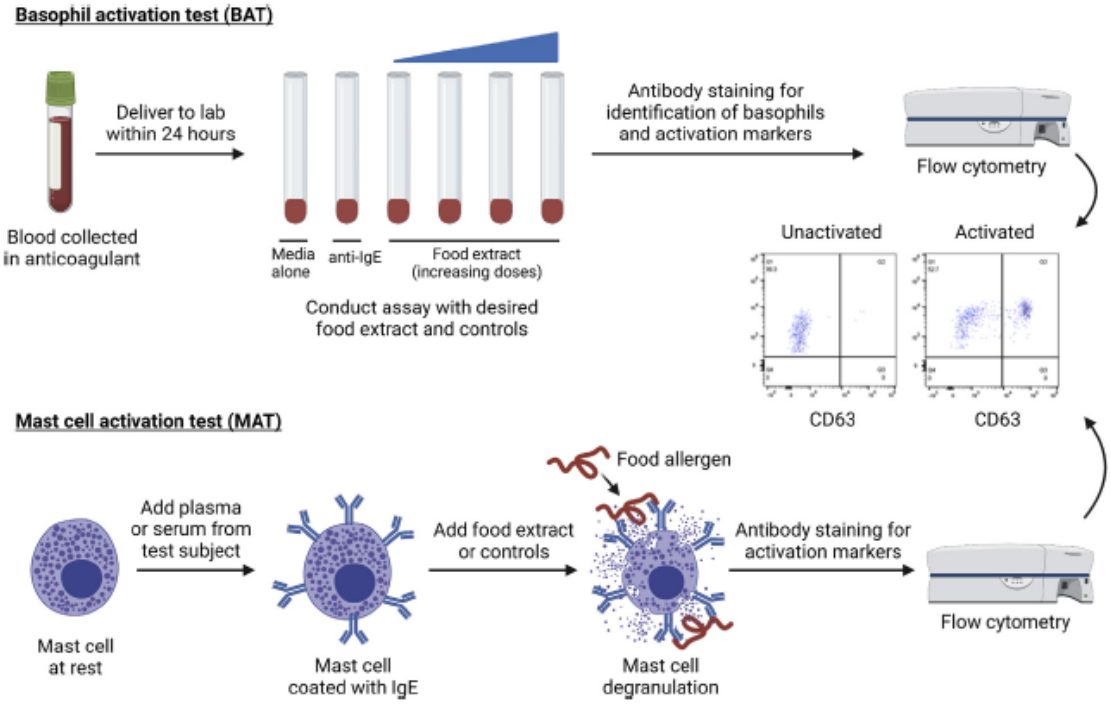
For this purpure a novel device (see Methods section) has been developed based on the measurement of the impedance values in serum samples that allows evaluating the allergic reactivity of a patient in response to a specific allergen by testing a patient's serum sample. As an advantage with respect to traditional tests in the new proposed method for determination of serum IgE, no antihuman IgE is needed, and only allergen and serum must be used. This technique is simpler compared to RAST/Immunocap because it minimizes the

possible loss of allergenic epitopes in the preparation of the solid phase, and it is more rapid and cheaper (only the allergen cost) than the immunoenzymatic one [106].

1.5.3 Basophil activation test (BAT) and mast cell activation tests (MAT)

More than the quantity of sIgE, the quality of sIgE is critical to determine the degree of mast cells and basophil activation, which is ultimately responsible for the allergic symptoms. Recently, Hemmings et al. [109] demonstrated that the proportion of IgE that is allergen-specific, the diversity of IgE response in terms of allergens that sIgE recognizes, the avidity for binding to the allergen, and sIgE levels all contribute to the proportion of mast cells and basophils that become activated in response to allergen stimulation. The beauty of cellular tests such as the BAT and the MAT is that they take all of the elements of sIgE into account when determining the likelihood that allergen exposure will lead to allergic reactions in an IgE-sensitized individual [110]. The BAT uses whole blood whereas the MAT uses plasma or serum to sensitize mast cells that can be either a cell line or primary cells derived from peripheral blood or from tissue. These basophils or mast cells are then stimulated with allergen and analyzed by flow cytometry to detect the expression of activation markers before and after stimulation with allergens or controls (Figure 5).

Figure 5. Overview of basophil activation test and mast cell activation test (from [111])



Both allergen extracts and individual allergens can be used for cell stimulation. Their diagnostic performance differs depending on the allergen source [110][112]. Generally, BAT has shown high sensitivity and specificity to diagnose peanut and other food allergies [70] [113][114]. The MAT has shown similar specificity in the diagnosis of peanut allergy but lower sensitivity, because this is a more artificial system requiring antibodies in a fixed volume of plasma to bind to receptors on the surface of mast cells, and it depends on the density of such receptors on the cell surface [115]. Because BAT and MAT require flow cytometry, with current methods it is not feasible to employ them as a screening test. Instead, they may be used as a confirmatory test, as a second step in the diagnostic workup in cases in which the first assessment with SPT or sIgE was inconclusive [83][116]. Applied this way, the high specificity is the main added value to the SPT and sIgE, which have high sensitivity but relatively lower specificity [70][113].

However, with more high-throughput methods, BAT, which also has high sensitivity, could be used more widely. Although BAT has high accuracy, approximately 10% to 15% of individuals with basophils who respond to non-IgE-mediated stimulants but not to IgE-mediated control or allergen (so called non responders) have an uninterpretable BAT [111]. For these people, MAT, which uses a passive sensitization system in which only the plasma comes from the patient, can provide a conclusive result [115].

1.5.4 Oral Food Challenge

The OFC remains the gold standard for a definitive food allergy diagnosis [19][71]. An OFC involves feeding the allergen in increasing quantities, 15–30 min apart, until the top dose is tolerated, or, in the case of a positive challenge, objective symptoms are observed [71] [117]. While generally considered safe, the OFC does pose a risk of a severe allergic reaction and as demonstrated in 2017, very rarely death [118]. OFCs can be open, single-blinded or DBPCFC, the latter being time and resource intensive, requiring both blinded and unblinded staff as well as active and sensory-identical placebo foods [119]. DBPCFC are recommended in research settings and are reported useful in routine clinical practice to reduce subjective symptoms in an anxious child, although OFCs are seldom stopped for subjective symptoms [4][71]. Open OFCs are suitable in the day-to-day clinical setting, with DBPCFC being used as an alternative in cases in which subjective symptoms or psychological aspects may hamper an accurate diagnosis using open OFCs. The demand for OFCs in infants is increasing due to the recent move towards the early introduction of allergenic foods [120][121]. Open challenges are considered appropriate in infants, both in the clinical and

in the research settings, as they have diminished ability to communicate subjective symptoms [118][122]. The suitability of the challenge food including the form, texture and volume needs to be considered in young infants who many have limited food intake [118]. There is no consensus on the number of doses or the ideal protein content for OFCs. The PRACTALL guidelines recommend a first dose of 3 mg increasing to a final dose of 3 g of protein reaching a cumulative dose of 4.443 g of food protein; however, the doses are not allergen specific and are not practical or representative of portion sizes for all foods particularly those with high and low protein content [71]. Alternatively, Nowak-Wegrzyn et al.[123], suggest that the cumulative dose should be representative of an age-appropriate portion of the food being tested, and therefore doses should be standardized by age. Prior to the OFC, the allergic child should be well, asthma-controlled and off any medications that can affect the interpretation or treatment of allergic reactions during an OFC, such as antihistamines, oral steroids, B-blockers, nonsteroidal anti-inflammatory drugs and ACE inhibitors [119]. Observations of the skin, respiration, oxygen saturation, heart rate and blood pressure need to be carried out before testing, and regularly throughout the OFC, to monitor for signs of an allergic reaction [71]. Those at risk of a severe reaction should be challenged in hospital by an experienced specialized clinical team with access to medications including antihistamines, b-agonists, corticosteroids, oxygen, fluids as well as intramuscular and intravenous adrenaline [117][124]. An experienced investigator is required to assess the outcome of the challenge [125]. OFCs are usually stopped with the development of objective symptoms however the decision is ultimately up to the attending physician. Following a positive OFC, allergen avoidance should be recommended, and an appropriate emergency treatment plan issued. Following a negative OFC, families should be advised to maintain regular consumption of the food to avoid a potential loss of tolerance to the food challenged [126].

1.6 Treatment

1.6.1 Allergen avoidance

Primary treatment of FA includes strict avoidance of responsible food and prompt identification and treatment of adverse reaction [4] Food avoidance represents the mainstay for preventing food-induced reactions in the long-term management of IgE- mediated FA. Patients and their families need to be instructed regarding food avoidance, underlining the importance of a strict adherence to the dietary indications provided, together with extreme

care in cross contact, safe storage, cleaning procedure as well as be careful to ingredients and food [84].

Food avoidance could have detrimental effects on nutrient intake, resulting in nutritional deficiencies [127][128] therefore nutritional counseling and growth monitoring are recommended for children and patients with single or multiple FA. FA has an impact on the quality of life of affected children and adolescents as well as their families and caregivers [129]. This type of attitude, which we could define as "passive", does not overcome the risk of accidental reactions due to the involuntary intake of the culprit food [130].

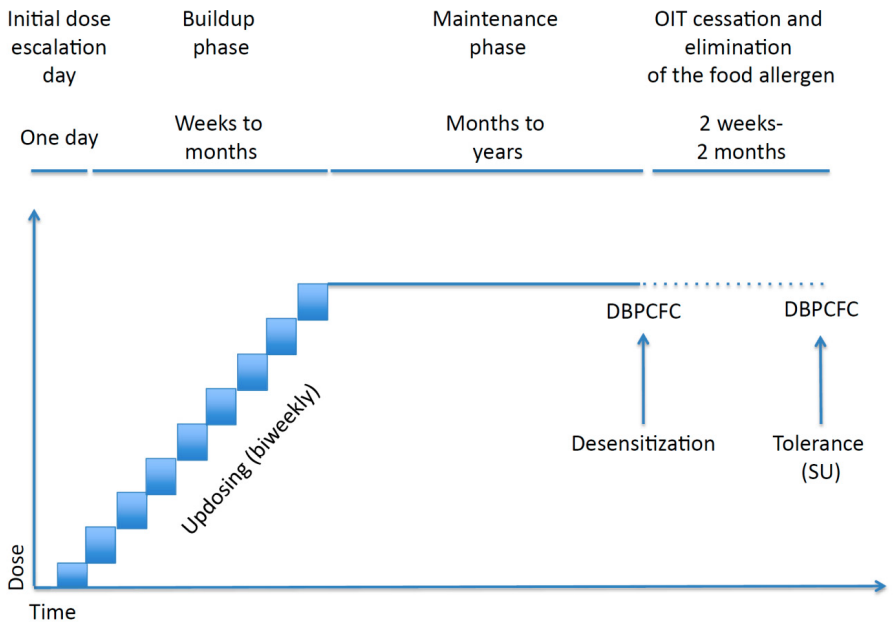
1.6.2 Allergen specific immunotherapy

Food-specific immunotherapy is becoming an "active" treatment option for IgE-mediate FA, actually used only for research purpose [130][131].

There are different types of immunotherapies, based on the administration route, and namely, oral immunotherapy (OIT) where the allergen is swallowed, sublingual immunotherapy (SLIT) where the allergen is held under the tongue for 2 min and then split or swallowed, and epicutaneous immunotherapy (EPIT), where a patch with a food allergen is applied on the skin [132].

The typical protocol of oral immunotherapy includes an initial escalation phase followed by dose build-up and maintenance phases, Figure 6.

Figure 6. Typical protocol of food oral immunotherapy (OIT) (from [133])



The initial escalation phase is often carried out on a single day and its purpose is to identify the starting daily dose for home administration. During the build-up phase, the daily dose is normally increased every 15 days until the maintenance dose is reached. At home, the patients should continue to take the same maintenance dose every day. After some months/one year, a DBPCFC is performed to verify tolerance. To assess a sustained unresponsiveness, the daily dose is then stopped for a period of 4 to 12 weeks and reintroduced during a DBPCFC. If no adverse reaction occurs, this state is defined permanent tolerance [133].

The aim of immunotherapy is to reach "a state of tolerance" in which the patient does not show any reaction after ingestion of a normal serving of the culprit food despite a period of absence of exposure. If the tolerance is not reached, we talk about desensitization, which indicates the ability to safely assume the culprit food, but it strictly depends on the daily intake of the same food [134].

The OIT induces desensitization (relative risk (RR) = 0.16 (95% CI 0.10–0.26)) but there is no evidence that oral immunotherapy induces long-term tolerance (RR = 0.29 (95% CI 0.08–1.13)) [132]. A study on egg oral immunotherapy conducted by Jones et al. [135] shows how the tolerance is enhanced with the duration of oral immunotherapy. The sustained unresponsiveness increases from 27.5% after 2 years of oral immunotherapy, up to 50% after 4 years. While the SLIT induces desensitization (RR = 0.26 (95% CI 0.13–0.64)), it is not as good as OIT [132]. In fact, in a study on SLIT for peanut, conducted by Burks et al. [136], the sustained unresponsiveness was reached in only 11% of the patients.

Regarding safety, the risk of systemic reactions is higher in those receiving OIT compared to placebo (RR = 1.16, (95% CI 1.03; 1.30)). The local reactions are mild, such as oral allergic syndrome or abdominal pain (RR = 2.14, (95% CI 1.47; 3.12)) [132].

Eosinophilic esophagitis (EoE) is a long-term side effect of OIT, and its prevalence in subjects undergoing OIT varies from 2.7% [137] to 30% [138]. This wide variability derives from the fact that not all patients who develop gastrointestinal symptoms during OIT undergo a gastroesophageal biopsy; therefore, with the risk of overestimating this side effect. A recent study [139] showed that OIT-induced EoE can be treated with a slower dosage regimen and a lower maintenance dose.

In SLIT, the systemic reactions are less frequent and milder, similar to a placebo arm (RR 0.98 (0.85–1.14)). The local reactions are frequent (7–40% of patients) and represented by

the oral allergic syndrome [132]. To date, there have been no cases of EoE developing during the SLIT for the management of food allergy.

An alternative immunotherapy route, to improve the safety of OIT, is epicutaneous immunotherapy. It consists of a patch, called Viaskin, which is applied on the skin. The allergen protein, adhering to the inside of its surface, is dissolved by the moisture from natural transepidermal water loss accumulated under the patch. The permeability of the stratum corneum, increased by the moisture collected under the patch, allows native proteins to concentrate near antigen-presenting immune cells. The upper skin is not vascularized; therefore, the systemic absorption of the allergen is almost eliminated [140] study on peanuts demonstrated that patients treated with Viaskin did experience a significant increase in a successfully consumed dose compared to the placebo group (placebo vs. VP100 (Viaskin peanut 100 mcg), $p = 0.014$; placebo vs. VP250 (Viaskin peanut 250 mcg), $p = 0.003$); in particular the study showed that younger children experienced a more favorable outcome ($p = 0.03$; age, 4–11 vs. >11 years) [78]. EPIT seems to be safe and well tolerated. The most frequent reaction is a local skin reaction at the application site [135].

A preliminary study on EPIT with milk failed to demonstrate a statistically significant improvement of the cumulative tolerated dose between the active group and the placebo group [141].

Another way to improve safety is the use of processed food, especially baked egg and milk. Thermal processing alters allergenicity via denaturation of the epitopes, or by altering susceptibility to digestion. Allergenicity may be further reduced via interaction with wheat proteins, in particular gluten, affecting solubility and bioavailability [142].

In baked-food reactive patients, the OIT with baked products is considered as immunotherapy, whereas in baked-food, tolerant patients, it is instead a marker of a milder, more transient allergy phenotype [143] In 2017, there was very weak evidence from a systematic review that baked egg and milk OIT could accelerate the acquisition of tolerance [144]. In 2018, a randomized controlled trial conducted on 84 patients with milk allergy demonstrated that a statistically significant higher percentage of patients who consumed baked products reached a tolerance to unheated milk versus patients not consuming baked products [145].

An anti-IgE monoclonal antibody was first proposed as an adjuvant to facilitate OIT by reducing allergic reactions induced by OIT [146]. There are studies on omalizumab and OIT with peanuts [147][148], egg [149], milk [150][151] and multiple foods [152]. All these

studies demonstrate that omalizumab enables faster achievement of the target maintenance dose and reduces the rate and severity of IgE-mediated reactions during oral immunotherapy. The use of omalizumab is off label for food allergy immunotherapy and its use is recommended for patients with severe food allergy who failed to be cured by oral immunotherapy [153].

A new therapeutic option, which showed promising results, is the use of probiotics with OIT. Tang et al. [16] has studied the co-administration of Lactobacillus rhamnosus with peanut oral immunotherapy in children with peanut allergies in a double-blind, placebo-controlled trial; 82.1% of patients in the active group compared to 3.6% of patients receiving placebo exhibited permanent tolerance within three weeks after stopping treatment. A follow-up study four years after discontinuing treatment found that the sustained unresponsiveness was maintained by 7 out of 12 patients in the active group versus 1 out of 15 in the placebo group [154].

In terms of food allergy treatment strategies, it remains unsatisfactory that, at the moment, the only effective treatment is the elimination diet. In addition to immunotherapy, the use of biologics also appears to be promising. However, more and larger clinical trials are needed to clarify the potential of these therapeutic strategies—such as combining administration routes to improve both efficacy and safety of immunotherapy. Moreover, the use of immunomodulatory agents is being developed and, depending on their results, they could become an important possible treatment for food allergies [43].

2. Objective

The aim of the present study is to provide an alternative and more efficient method for diagnosis of cow's milk allergy (CMA), one of the main FAs in children, by measuring the impedance values of a patient's serum sample before and after its contact with an allergen of interest, in this case CM casein. For this purpure a novel device (see Methods section) has been developed based on the measurement of the impedance values in serum samples that allows evaluating the allergic reactivity of a patient in response to a CM casein by testing a patient's serum sample in a very easy and fast way.

3. Material and Methods

This prospective study enrolled children with a personal history of reaction to CM as well as children without history of CMA (control group) referred to Allergy Unit of the Meyer

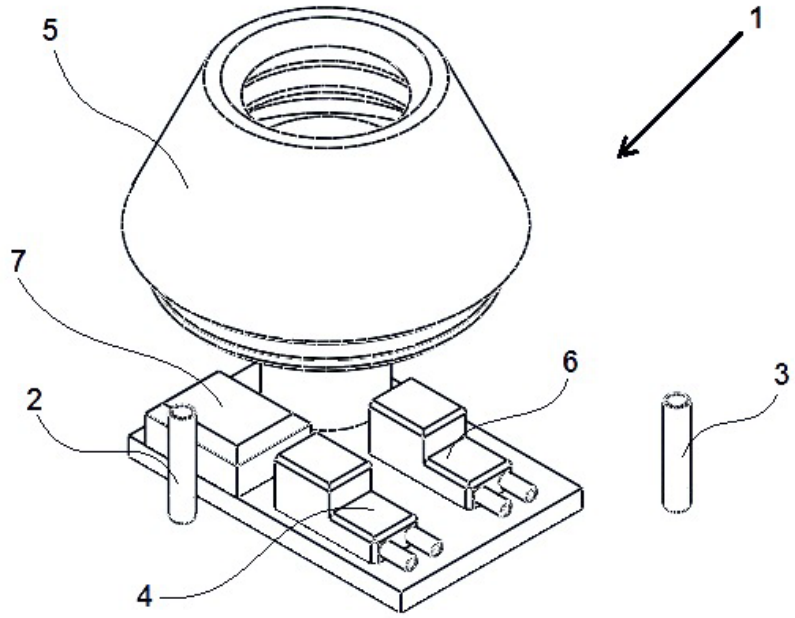
Children's Hospital, Florence, Italy (from September 2019 to September 2020). The SPT to CM and CM casein, using respectively fresh milk and commercial extracts (0.1 mg/mL, Lofarma, Milan, Italy), were performed. Positive and negative controls were used: histamine (10 mg/mL; Lofarma, Milan, Italy) and normal saline, respectively. The results were read after 15 minutes: a largest wheal diameter ≥ 3 millimetres (mm) was considered positive. Blood samples were collected to determine s-IgE to CM and CM casein using an ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). A positive cut-off point was set at 0.1 kUA/L. An aliquot of blood sample was reserved for impedenzimetric measurement. Patients with positive SPT or detectable CM casein-IgE underwent to oral challenge with baked milk. Baked milk challenges were performed as open challenges under physician supervision. Parents will be instructed to prepare cakes at home according to a specific protocol that our clinic provided. Each cake contains 1.3 grams of milk protein from non-fat dry milk powder. The cakes were baked at 180°C in an oven for 30 minutes. A standard graded open food challenge consisted in increasing increments every 20 minutes of 1/4 (325 mg), 1/2 (650 mg), and 1 + 1/4 (1,625 mg) cake, totalling 2.6 grams of milk protein. Subjects were monitored throughout and for 120 minutes after the completion of the challenge. Challenges were discontinued at the first objective sign of reaction [9], and treatment were initiated at the discretion of the supervising physician. According to Niggeman's classification, the OFC clinical manifestations were classified as mild, moderate or severe [10] and consequently the CM allergic patients were classified in different severity degree, in particular highly allergic (HA), medium allergic (MA) and low allergic (LA) children.

For the impedenzometric measurement an impedance measurement device was used, described by Barni S et al. [11]. The device, represented in Figure 7, is composed by:

- a container 2 for CM casein for which specific IgE are to be detected in a patient's serum sample;
- a measurement unit 3 comprising an appropriate support for allergen/serum samples and electrodes for the generation of AC electrical signals on the support;
- a pump 4 in order to push an allergen sample from the container 2 and pumping it towards the measurement unit 3 through suitable pipes;
- a container 5 for a patient's serum to be tested, optionally comprising a centrifuge and filtering device for the treatment of the patient's whole blood and its separation for obtaining a serum sample to be tested;

- a pump 6 for taking a patient's serum sample to be tested from the container 5 and pump it towards the measurement unit 3 through suitable pipes;
- a processing unit 7 associated to the measurement unit 3 can collect and process the data coming from said unit 3 to obtain corresponding impedance values of the samples tested and send them to a display or to a remote station for a further processing and display of data.

Figure 7. A novel impedance measurement device



The impedance measurements are carried out in AC regime at a frequency ranging from 10 Hz up to 100 kHz with a resolution of at least 1 Hz, so as to minimize the interaction and the biasing effects of those substances still present in serum, that could become polarized if biased with a constant voltage; in this way the properties of the serum under test are preserved at highest grade.

The flow of serum and allergens in the device of the invention are assisted by pumps that let then flow through pipelines and regulate their deposition on the support in the measurement unit 3. The amount of serum to be tested and of allergen in the present device is regulated to be from 100 uL to 40 mL. The impedance values measured for such volumes can range from 500 kΩ to 5 Ω. A dedicated software in the processing unit 7 can set the proper measurement parameters to perform the impedance measurements in a range of frequency to perform a frequency sweep or at a single frequency according to the test needs, and the amplitude of the signals on the sample may be set for instance

between 200mVpp and 2 Vpp.

The data collected from the measurements can then be collected through a shared or dedicated bus; preferably, a I2C communication protocol is used to set up the impedance measurement unit and the collection of data obtained.

The present method for in vitro diagnosis of allergic disease comprises the following steps:

-contacting a CM casein containing solution with a support provided with electrodes;

-measuring impedance of CM casein containing solution, obtaining a first impedance value;

-adding a patient's serum to CM casein containing solution on to said support provided with electrodes;

-measuring impedance of patient's serum added to CM casein containing solution, obtaining a second impedance value;

-evaluating the difference between said second and said first impedance values, this difference being correlated to the concentration of IgE specific to said allergen in said serum.

The above said first impedance value obtained for the CM casein containing solution represents a baseline of the present measurement method, corresponding to the base impedance at the frequency and amplitudes selected for the measurement. The variation of impedance when patient's serum is added is correlated with the concentration of IgE present in serum and specific to the CM casein and can gives a measure of the interaction between the CM casein and the specific IgE.

The measurement phase consists of performing an impedance measurement at a fixed frequency sinusoidal signal (@30 kHz) each 128 μs, starting when the serum and the allergen come into contact until the steady state is reached and then performing a frequency scan (in the range 1–90 kHz), providing the impedance spectrum of the MUT at the end of the serum-allergen reaction.

4. Results

Overall, 33 patients were enrolled: 15 children with CMA and 18 not-CM allergic children. The clinical and allergologic characteristics of children with CMA are shown in Table 1.

Table 1. The clinical and allergologic characteristics of children with CMA

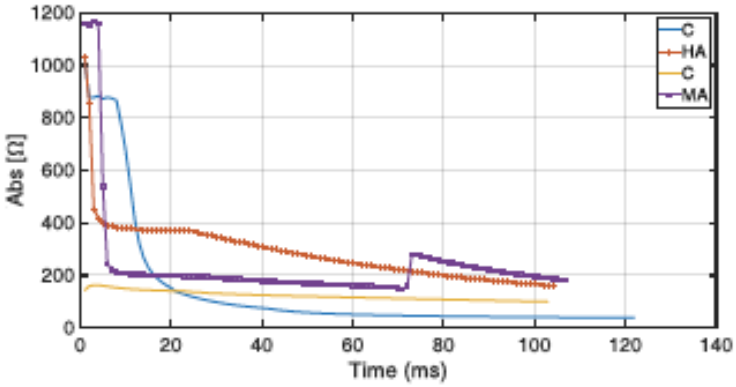
N°, sex	SPT to CM (mm)	SPT to CM casein (mm)	S-IgE to CM (kU/l)	S-IgE to CM casein (mm)	Symptoms during OFC	Trigger dose of CM (ml)	CMA severity degree
1, M	12	5	>100	>100	C, R, U	8.1	HA
2, M	12	5	>100	>100	C	0.1	HA
3, F	3	2	>100	>100	AP, U	0.75	HA
4, F	8	5	>100	>100	U	1	HA
5, M	8	4	>100	>100	V, R, U	2.1	HA
6, F	10	8	60	49.2	C, R, V	6	MA
7, F	10	8	52	41.7	R, S	12	MA
8, F	13	5	61.8	56	R, U, Co	9,6	MA
9, M	7	3	55.2	40	U, R, C	11	MA
10, M	6	2	52.5	44.8	AP	45	MA
11, F	5	6	30.5	21.1	U, A, C, S	8.1	LA
12, M	7	2	27.8	21.1	C, Co	30	LA
13, M	4	2	20.6	17.6	C, R, Co	26.4	LA
14, F	10	8	30.5	27.2	C, U	1.8	LA
15, F	10	10	25	20.9	C, U	2.4	LA

Legend: A=angioedema; AP=abdominal pain; C=cough; CM: cow's milk; Co=conjuntivtis; HA=highly allergic; LA=low allergic; MA=medium allergic; mm=millimiters; OFC=oral food challenge; R= rhinitis; S=sneezing; SPT=skin prick test; U=urticaria; V=vomiting

Table 1. The clinical and allergologic characteristics of children with CMA

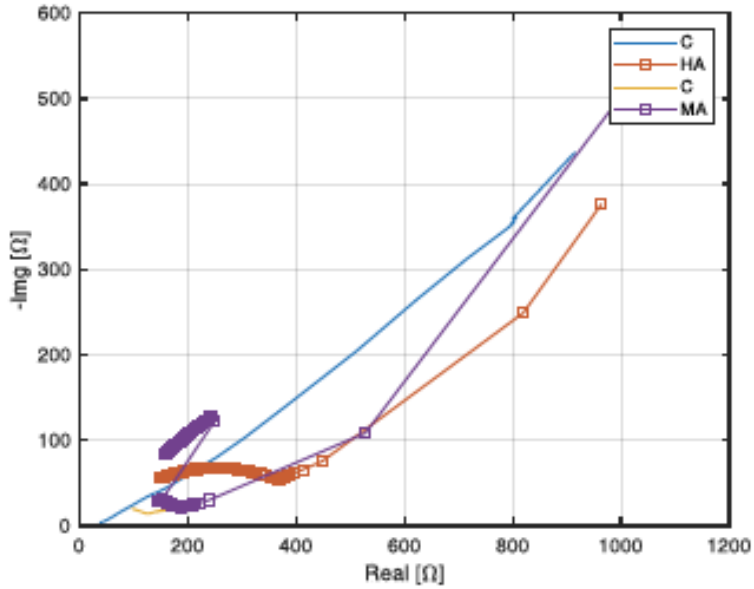
The Figure 8 shows the time results of the first bunch of children samples collected (f@30kHz). The two solid lines represent the controls while the red and purple curves are two HA patients. It can be noticed that the starting values as well as the final resistance ones are highly differentiated allowing for severity discrimination. The step variation in the purple curve represents a random single measurement error due to fluids oscillation in the sample holder which can be deleted and avoided.

Figure 8. Time variation of the absolute value of the impedance at fixed frequency of the first bunch of samples



In Figure 9 the same measurements are represented in an impedance spectroscopy (IS) diagram which allows to discriminate between the allergic and control samples in the last part of the measurement corresponding to the high frequency (80-90 kHz) part of the measurement.

Figure 9. IS diagram of the first bunch of samples spanning the range 1kHz- 90kHz



The Figure 10 shows the second bunch of HA casein allergic candidates compared with a higher number of controls. It is evident from measurement that the resistance values vary from 1.2kΩ to150Ω. The lines with markers of Figure 10 represent the HA subjects and again it is evident that it is possible to identify them in a straight way with respect to the bundle of controls. In particular some parameters which are characteristic of allergic subjects can be identified and exploited to build a characteristic feature signature to be used in a subsequent classifier for automatic identification and cutoff determination. These parameters can be summarized in the normalized initial value, in the slope taken in between samples

(between sample 10th and 20th divided by the sample number), the second distance measure, intended as the difference between the absolute value sample taken at the 50th sample point of the curve under analysis with respect to the reference control established for the specific trial and the most meaningful which is the final value at the end of the measurement. Together with the time plot indicators it is possible to highlight as shown in Figure 11 that the IS diagram provides useful information.

It is actually possible to identify two different areas in the plot where, focusing the attention, differences between allergic subject and control may arise.

Figure 10. Time variation of the absolute value of the impedance at fixed frequency of the second bunch of samples

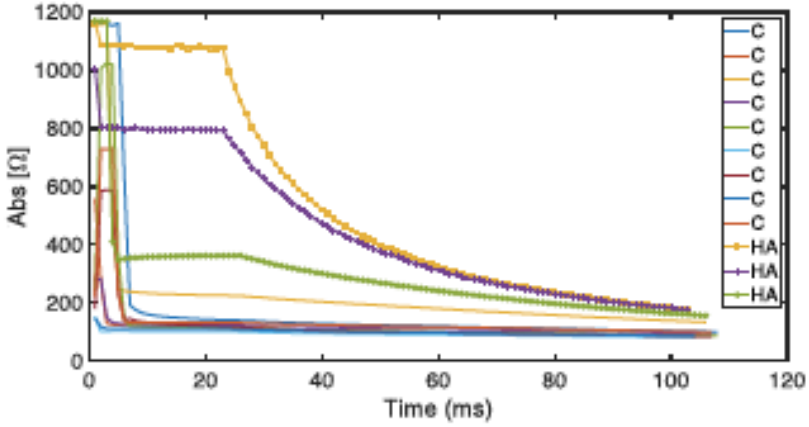
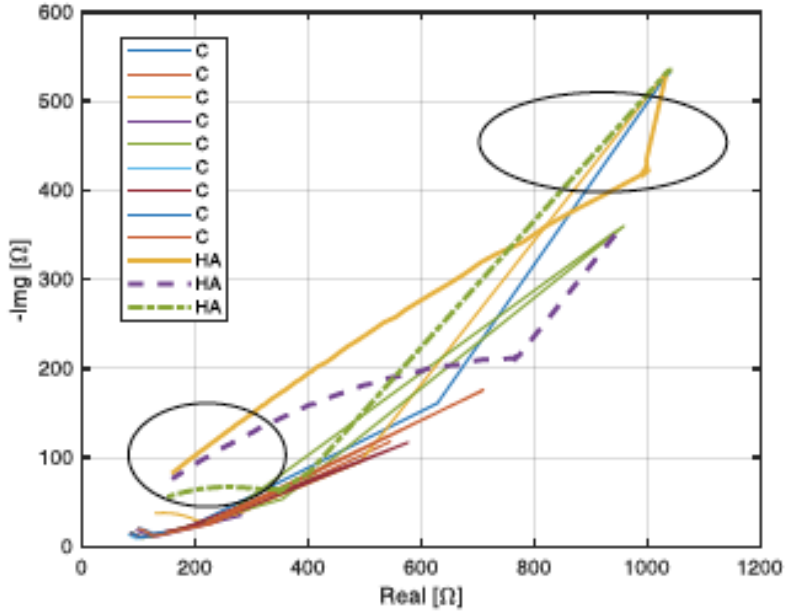
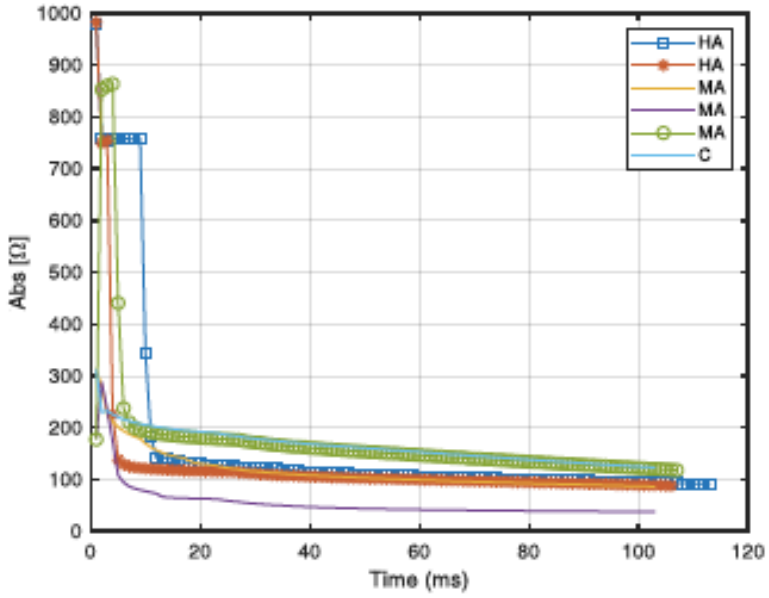


Figure 11. IS diagram highlighting the differences between areas belonging to allergic subject and control



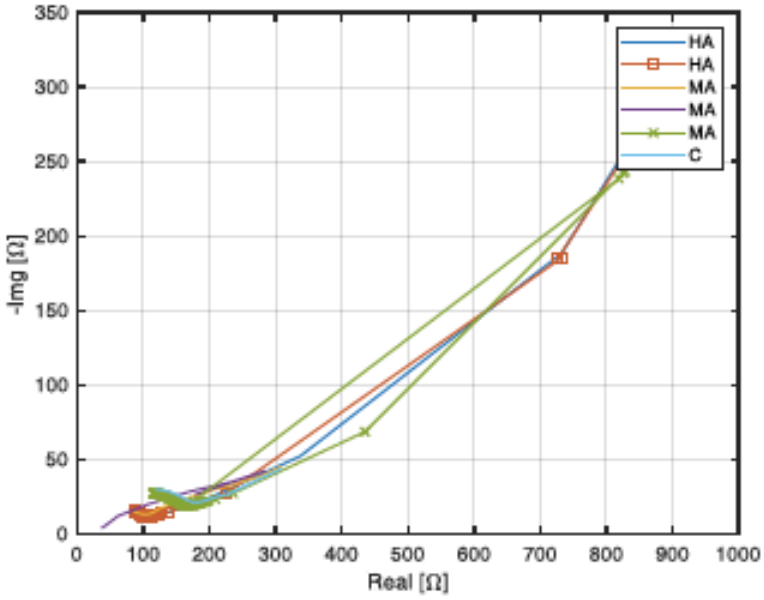
In Figure 12 some other samples comparing not only HA children but also MA children with a single control, are shown. Even in this case it evident that the allergic units can be identified, and it is furthermore possible to discriminate among HA and MA samples focusing on the previously mentioned parameters set.

Figure 12. Time variation of the absolute value of the impedance at fixed frequency comparing HA and MA subjects



In Figure 13 the corresponding IS diagram is shown and in the high frequency region it is possible to find again useful information for allergic patients' identification.

Figure 13. IS diagram showing the results for HA and MA patients



In Figure 14 it is possible to compare with a single control subjects with different HA who are affected by different HA severities ranging from 70 to 100 kU/L as Unicap indicator. In figure 15 it is shown its corresponding IS diagram which allows to identify clearly the behavior of the control samples at high frequency.

Figure 14. Time variation of the absolute value of the impedance at fixed frequency comparing values for the Unicap indicator

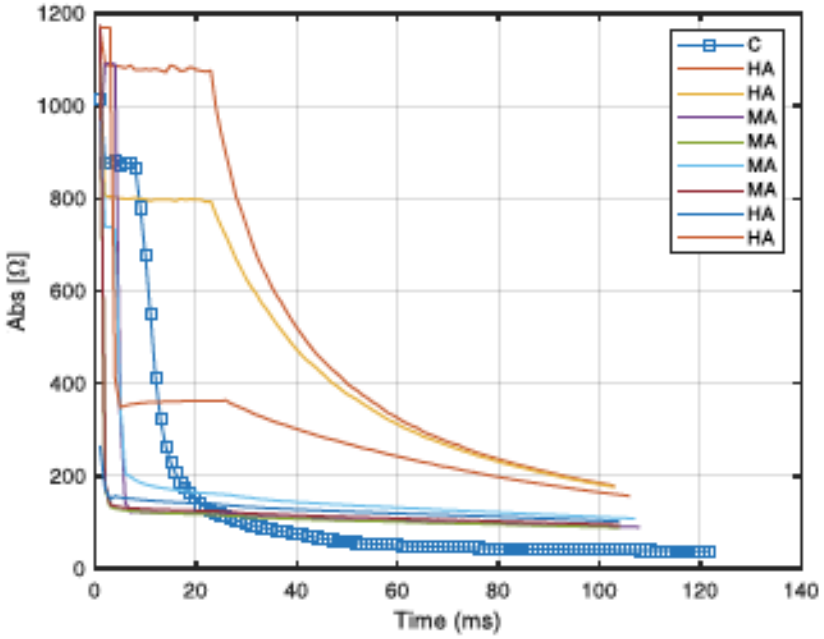
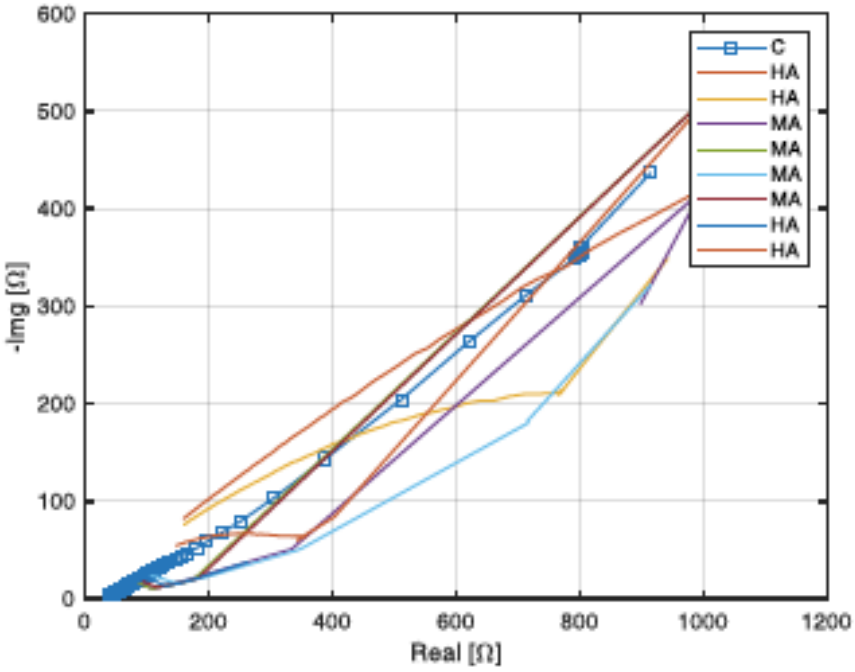


Figure 15. IS diagram showing the results for Unicap indicator values



Finally in Figure 16 and 17 (IS) an additional sample test has been conducted on two subjects who have a very close HA allergic degree (90 and 100 respectively) and compared with some controls. The curves especially in the end values agree with the expectations and are fully differentiable from the controls especially in the decreasing time constant.

Figure 16. Time variation of the absolute value of the impedance at fixed frequency comparing values for two subjects having close HA allergic degree

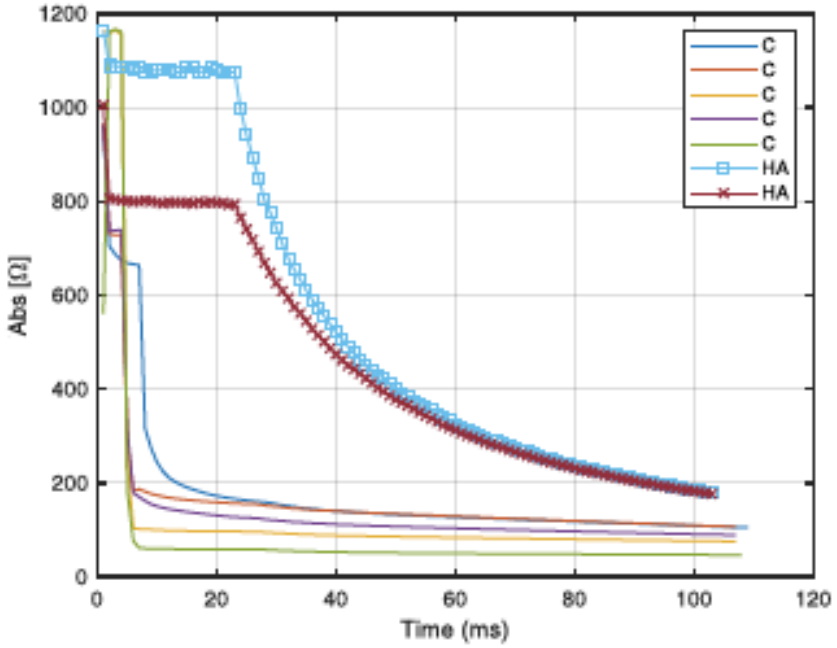
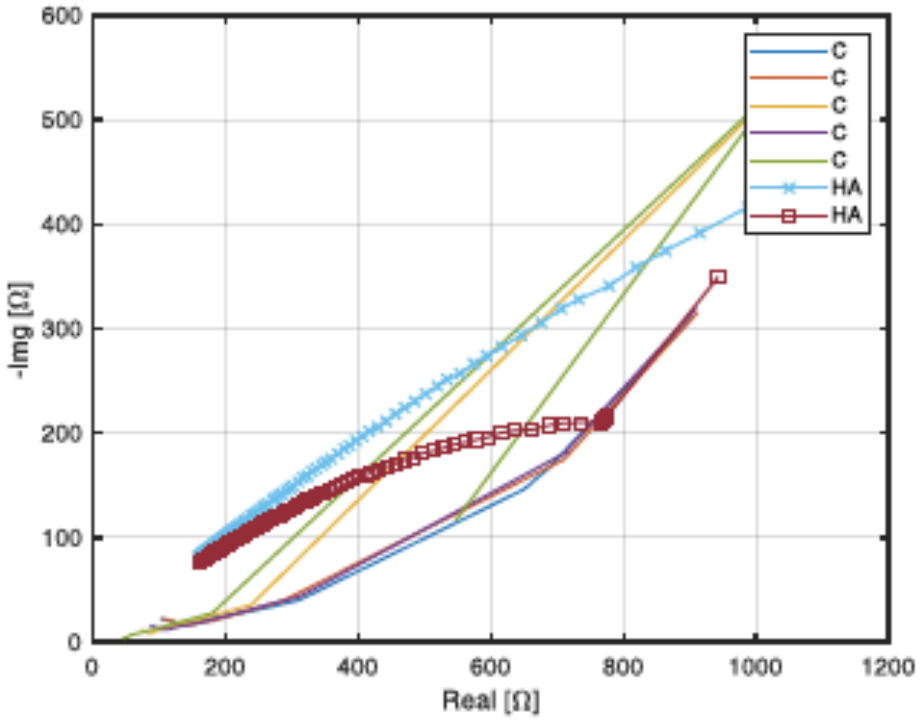


Figure 17. IS diagram showing the results for close HA allergic degree



Of course, performing measurements on biological samples is not an easy task due to the peculiarity of blood samples in this case. Variability has been reduced working on serum instead of whole blood, nevertheless uncertainty introduced by sample size variability can be controlled only reducing at minimum the sample size.

To allow for a direct result in the effort to find a sort of cut-off synthesis threshold a Fuzzy logic network has been designed (Figure 18) exploiting the classical Mamdani classification approach (IF-THEN approach) with five input parameters extracted by the time curves only and described in terms of significance and range according to Table 2.

The classifier has been applied on normalized input curves in order to reduce the parameter variation.

Figure 18. Fuzzy logic network scheme

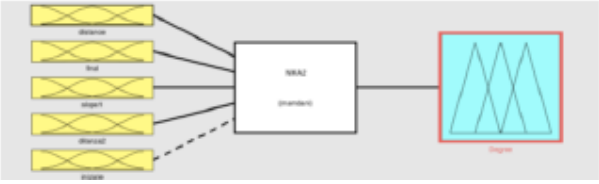
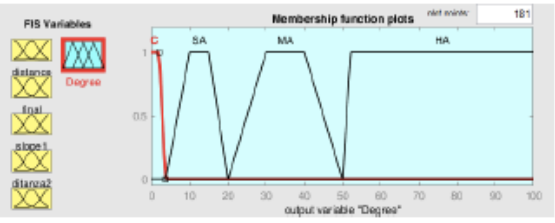


Table 2. Features for time domain graphs

Fuzzy Logic Synthetic Features for Time Domain Graphs		
Time Diagram	***Significance***	***Range***
Distance	Medium	0-1
Final	High	0-1
Slope1	Medium	0-1
Distanza2	Low	0-1
Iniziale	Low	0-1

The membership functions of both input and output have been selected as the one represented in Figure 19.

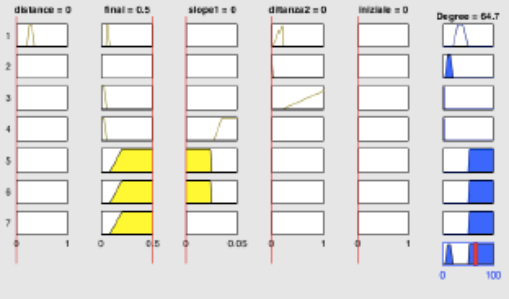
Figure 19. Output Membership functions plot



Finally, in Figure 20 the output for a specific input where the final value reaches 50% of the normalized output and which represent therefore a HA patient is shown to verify that the classifier can correctly identify the allergic subjects. Of course, more efficient classifiers

could be implemented and the reason of the author's choice lays in the fact that uncertainty in the measurement acquisition of biological as well as physiological samples is significative and crisp classifiers may be limited.

Figure 20. Example of the classifier output for a specific input, showing HA patients



5. Discussion

In this study we presented some results on a representative number of cases taken among children which are affected by different casein allergy severity degrees. In particular 3 groups of highly allergic, medium allergic and low allergic children have been identified together with controls.

Two diagrams are presented as measurement synthesis: the time variation at a fixed frequency and the impedance spectroscopy one. Both provide signature components to discriminate between allergic children and components. The time dependent diagram provided information on the avidity of IgE protein with respect the allergen of interest (CM casein). This justifies why some children resulting allergic according to the Immunocap test to casein allergens do not show anaphylactic reaction while others with very limited allergic results and IgE total counts present heavy reactions instead. Indeed, s-IgE concentrations do not accurately predict the severity of allergic reaction but do reflect the likelihood of an allergic reaction of variable intensity. IS diagrams suggests that this behavior can be investigated at low frequencies and at high frequencies comparing results with controls. Such features have been then used, concerning the time graphs to feed a Fuzzy classifier in the effort to prove that a cut-off threshold can be experimentally identified.

This new proposed method for the determination of s-IgE has an advantage in comparison to the previous mentioned tests (RAST/Immunocap): no anti-human IgE are needed and only allergen and serum should be used. Moreover, this technique is simpler compared to RAST/Immunocap because it minimizes the possible loss of allergenic epitopes in the preparation of the solid phase, and it is more rapid (entire test takes 5 minutes for completion) and cheaper (only the cost of the allergen) than the immunoenzymatic one.

Therefore, this new proposed approach improves the performance of the current test for s-IgE concentration in terms of assay time and, furthermore, provides information about the IgE binding kinetics that is related to the severity of the allergic reactions. Our results, although promising, need to be confirmed by more experiments.

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