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MILK AND EGG ALLERGIES IN CHILDREN. IMMUNOLOGICAL CHANGES AND MECHANISMS UNDERLYING ORAL IMMUNOTHERAPY

ALERGIA A LA LECHE Y AL HUEVO EN NIÑOS. CAMBIOS INMUNOLÓGICOS Y MECANISMOS ASOCIADOS AL TRATAMIENTO CON INMUNOTERAPIA ORAL

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A mis padres, a mi hermano

"La vida es como montar en bicicleta.

Para mantener el equilibrio hay que

seguir pedaleando".

-Albert Einstein

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ABSTRACT

Food allergy is a major health problem in the Western countries, notably in children, with an estimated prevalence of 2-8%, and is the most frequent reason for anaphylactic reactions at this age. Between the food allergies, Immunoglobulin E (IgE)-mediated cow's milk protein allergy (CMPA) and egg allergy are the most common in infants, affecting approximately 1-3% of children. Although these allergies are associated with a high rate of natural tolerance, the mechanisms of tolerance acquisition are not well understood. Moreover, about 50% of children do not overcome their allergy within the first years of life, which rekindle in the increasing incidence of the clinical disorder in adults. These food-allergic processes may endanger children's health causing reactions from mild atopic dermatitis to severe systemic anaphylaxis, which can be life-threatening. Unfortunately, at present, the only treatment for cow's milk (CM) and egg allergies is a complete dietary restriction of implicated foods. Because of milk and egg protein is included in a wide range of cooked and manufactured foods, avoidance involves a wide dietary restriction, which leads to negative nutritional, social, psychological and economic consequences. However, and despite its importance, little information is known about which specific immune mechanisms constitute differential factors for the development of allergy or for the maintenance of tolerance. Consequently, new studies to clarify these important issues and novel strategies for immune intervention are currently pursued.

Given this scenario, the first research conducted in this thesis compiled an extensive analysis of immune cell subsets and cytokine secreting cells in infants with symptoms compatible with CMPA, which is the first allergy to appear in children. Samples were collecting in the 1-4 days after the first adverse reaction, to decipher the immune alterations related with the establishment of this allergy. Interestingly, results revealed that children who developed CMPA had decreased regulatory T cell (Treg) counts and lower serum vitamin D levels. Furthermore, these parameters were statistically correlated and constituted good predictors to distinguish between healthy controls and CM allergic infants.

Therefore, they could be crucial factors behind the onset of the allergic process in infants and a therapeutic target for the treatment of this food allergy.

Because of oral immunotherapy (OIT) is nowadays one of the most promising approaches toward a treatment for food allergy, further studies to lend more scientific evidence about such treatment are demanded. OIT involves giving regular, gradually increased dosages of the allergenic protein under medical supervision. The goal is induce desensitization firstly, defined as an increase in the threshold for reactivity but requiring a continued consumption of the allergen to prevent the reappearance of reactivity; and a later induction of oral tolerance, which means a long-lasting unresponsiveness against the food allergen. Pilot studies have yielded promising results, with success ratios frequently higher to 70%. However, differences between protocols employed, and the lack of knowledge about the specific immune mechanisms responsible of the desensitization and tolerance acquisition, prevent from drawing robust conclusion and make difficult the improvement and further development of this therapeutic strategy. Moreover, there is no yet evidence enough in the biomarkers that reflect the success of the intervention, as well as which children could be good candidates for treatment and have a reduced risk of adverse reactions.

In this Thesis, the clinical efficacy and immunologic changes associated with OIT for IgE-mediated CMPA and egg allergy in infants were evaluated. The basal immunologic status of the allergic children enrolled was assessed through comparison with those of a non-allergic group of the same age range and sex. Three different OIT schedules were evaluated: i) a rush protocol for egg desensitization based on a first 5-days rapid up dosing, with a rate of success (defined as the ability to eat one undercooked egg) of 93.8% of patients in 5 months of intervention; ii) a long-course regimen for egg desensitization, which allowed 60% children to eat the equivalent to a full egg (≥ 32 mL of pasteurized egg white) in an average period of 11.75 months; iii) a long-course OIT protocol for CM desensitization with 70% success (≥ 200 ml of CM) and, an average duration of 18.9 months. A distinct feature of the long protocols is the progressive introduction of egg or milk-containing foods into the patient's diet. Moreover, the long-term efficacy of

desensitization of these protocols was evaluated 24-48 months after being completed, reporting that 70-75% of the allergic children participants were consuming egg or CM as a part of their diet.

Analysis of immunological outcomes underlying OIT studies showed a decrease in serum allergen-specific IgE levels along the therapy, accompanied by a rise in the allergen-specific IgG4. Because the mechanisms by which OIT acts include modulation of T-cell responses, peripheral blood mononuclear cells (PBMCs) were isolated from blood samples and stimulated with ovalbumin (OVA) or β-casein (β-CN) for measuring T helper 2 (Th2), T helper 1 (Th1) and regulatory T (Treg) cells cytokine profile, as well as the expression of the master transcription factors of the corresponding T-cell differentiation (GATA3, T-bet and FoxP3). Results revealed a diminished allergic Th2 response in children successfully desensitized, with lower specific interleukin (IL)-13 and IL-5 production. Gene expression differences were not large enough to consider a significant change in either of the transcription factors studied. Higher baseline antigen-specific IgE levels are proposed predictors of a negative clinical response to OIT.

In summary, main findings in this thesis highlighted that circulating Treg cells and serum vitamin D levels could be crucial factors behind the establishment of CMPA in infants. Oral rush immunotherapy protocols could be highly effective in inducing desensitization to egg proteins in few days with a long-term protection. Successful desensitization resulted in significant reductions in antigen-specific IgE with increases in antigen-specific IgG4 and a drop in Th2 cytokines associated with allergic processes.

RESUMEN

La alergia alimentaria es un grave problema de salud en Occidente, especialmente en niños. Con una prevalencia estimada del 2-8%, es la causa más frecuentemente de las reacciones anafilácticas a dicha edad. La alergia a la proteína de la leche de vaca (APLV) y la alergia al huevo mediadas por inmunoglobulina E (IgE), son las alergias alimentarias más frecuentes en la edad infantil, afectando aproximadamente al 1-3% de los niños. Aunque estas alergias presentan una elevada tasa de resolución o tolerancia espontánea, cómo se produce este fenómeno no se conoce en profundidad. Además, alrededor del 50% de los niños no revierten su alergia durante los primeros años de vida, lo cual repercute en una creciente incidencia de la enfermedad en la edad adulta. Los procesos alérgicos implicados pueden poner en peligro la salud de los niños, causando reacciones que van desde la dermatitis atópica leve a la anafilaxia sistémica grave, condición que pone en peligro la vida del paciente. Desafortunadamente, en la actualidad el único tratamiento para la APLV y la alergia al huevo es la total restricción en la dieta de los alimentos implicados y, puesto que las proteínas de la leche y del huevo se incluyen en una extensa gama de alimentos cocinados y manufacturados, su eliminación implica una amplia restricción dietética, con consecuencias nutricionales, sociales, psicológicas y económicas adversas. Sin embargo, y a pesar de su importancia, poco se sabe acerca de los mecanismos inmunológicos específicos que conducen al desarrollo de alergia o a la preservación de la tolerancia. Por ello, es importante realizar estudios que permitan aclarar estas cuestiones, así como plantear estrategias innovadoras de intervención en dicha respuesta inmune.

Para intentar dar respuesta a estas interrogantes, la primera investigación realizada en la tesis doctoral consiste en un análisis exhaustivo de subpoblaciones celulares del sistema inmune y células secretoras de citoquinas en niños con síntomas compatibles con la APLV. Las muestras se recolectaron entre 1 y 4 días tras la primera reacción adversa, para poder estudiar las alteraciones inmunológicas relacionadas con el establecimiento de esta alergia. Los resultados revelaron que los niños que desarrollaron APLV tenían

menores recuentos de células T reguladoras, así como niveles más bajos de vitamina D en suero. Además, estos parámetros estaban estadísticamente correlacionados y eran buenos predictores para distinguir entre controles sanos y niños alérgicos. Por lo tanto, estos factores podrían tener una implicación crucial en el inicio del proceso alérgico en los niños y ser una diana terapéutica para el tratamiento de esta alergia alimentaria.

La inmunoterapia oral (ITO) es una de las estrategias actuales más prometedores para el tratamiento de la alergia alimentaria, por ello es prioritario que se realicen más estudios que arrojen una mayor evidencia científica sobre este tipo de tratamiento. La ITO implica la administración de dosis regulares, gradualmente crecientes de la proteína alergénica bajo supervisión médica. El objetivo es inducir un primer estado de desensibilización, definido como un aumento del umbral de reactividad, pero que requiere el consumo continuo del alérgeno para evitar la reaparición de la alergia; y una posterior inducción de la tolerancia oral, que significa la falta de respuesta duradera contra el alérgeno alimentario. Los estudios piloto han arrojado resultados prometedores, con tasas de éxito que superan el 70%. Sin embargo, las diferencias entre los protocolos empleados, y la falta de conocimiento sobre los mecanismos inmunológicos responsables de la adquisición del estado de desensibilización y de la tolerancia, impiden sacar conclusiones definitivas y dificultan la implementación de mejoras para el desarrollo de esta estrategia terapéutica. Por otra parte, todavía no hay suficiente evidencia en los biomarcadores que reflejen el éxito de la intervención, así como en los que indiquen qué niños podrían ser buenos candidatos para el tratamiento y cuáles tienen un riesgo menor de sufrir reacciones adversas.

Durante la realización de esta tesis doctoral se ha evaluado la eficacia clínica y los cambios inmunológicos asociados con la ITO para la APLV y la alergia al huevo mediadas por IgE en lactantes. El estado inmunológico basal de los niños alérgicos reclutados fue comparado con el de una población sana de igual rango de edad y sexo. Se evaluaron 3 protocolos diferentes de ITO: i) un protocolo rápido de desensibilización al huevo basado en una fase rápida de 5 días de duración, con una tasa de éxito (definida como la

capacidad de consumir un huevo poco cocido) del 93,8% de los pacientes a los 5 meses de intervención; ii) un régimen de larga duración para la desensibilización al huevo que permitó que el 60% de los niños comieran el equivalente a un huevo completo (≥ 32 mL de clara de huevo pasterizada) en un periodo medio de 11,75 meses; iii) un protocolo de desensibilización a la leche de vaca de larga duración que alcanzó un 70% de éxito (≥ 200 mL de CM) en un tiempo promedio de 18,9 meses. Una característica distintiva que fue estudiada en los protocolos de larga duración, es la introducción progresiva en la dieta del paciente de alimentos que contenían leche/huevo. Por otra parte, la eficacia de desensibilización a largo plazo fue evaluada a los 24-48 meses tras completar el protocolo, reflejando que un 70-75% de los niños alérgicos participantes mantenían el consumo de huevo o leche de vaca como parte normal de su dieta.

Tras el análisis de los resultados inmunológicos subyacentes a los estudios de ITO, se observó una disminución en los niveles séricos de IgE alérgeno-específica a lo largo de la terapia, acompañada de un aumento en la IgG4 específica a alérgenos. Dado que los mecanismos sobre los cuales actúa la ITO incluyen la modulación de respuestas de las células T, las células mononucleares de sangre periférica de las muestras de sangre fueron aisladas y estimuladas con ovoalbúmina (OVA) o β-caseína (β-CN) para medir la secreción de citoquinas asociada a la respuesta de linfocitos T colaboradores Th2, Th1 y células T reguladoras (Treg), así como la expresión de los genes maestros controladores de las correspondientes diferenciaciones de dichas células T (GATA3, T-bet y FoxP3). Los resultados obtenidos mostraron una disminución de la respuesta alérgica Th2 en niños desensibilizados satisfactoriamente, con una menor producción de interleucina (IL)-13 e IL-5. No se observaron cambios significativos en la expresión génica en ninguno de los genes estudiados. Los resultados indican que, niveles más elevados de IgE específica a alérgenos al inicio podrían servir para predecir una respuesta clínica negativa al tratamiento.

En resumen, los principales hallazgos de esta tesis señalan que la cantidad de células Treg circulantes y los niveles séricos de vitamina D podrían tener un papel crucial en el

establecimiento de la APLV en los niños. El protocolo rápido de inmunoterapia oral se mostró muy eficaz a la hora de inducir la desensibilización a las proteínas del huevo en pocos días, con una protección que se mantuvo a largo plazo. El éxito en la desensibilización se asoció con reducciones significativas de IgE específica a alérgenos, con aumentos de la IgG4 específica y con una disminución en las citoquinas Th2 asociadas con los procesos alérgicos.

ABBREVIATIONS

DBPCFC: Double blind placebo controlled food challenge

CM: Cow's milk

CMPA: Cow's milk protein allergy

EW: Egg white

IL-: Interleukin-

IFN-γ: Interferon-γ

LZ: Lysozyme

OIT: Oral Immunotherapy

OM: Ovomucoid

OVA: Ovalbumin

PBMCs: Peripheral blood mononuclear cells

ROIT: Rush Oral Immunotherapy

sIgE: Specific Immunoglobulin E

sIgG4: Specific Immunoglobulin G4

SPT: Skin prick test

TNF-α: Tumor necrosis factor-α

Treg: Regulatory T cell

α-LA: α-Lactalbumin

β-CN: β-Casein

β-LG: β-Lactoglobulin

1. INTRODUCTION

1.1. FOOD ALLERGY IN THE PEDIATRIC AGE

1.1.1. Food allergy – an immune mediated adverse reaction to food

There is no universally accepted definition for food allergy. The expert panel of the National Institute of Allergy and Infectious Diseases of USA (NIAID) defines food allergy as "*an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food and is distinct from other responses to food, such as food intolerance, pharmacological reactions, and toxin-mediated reactions*" (Boyce et al., 2010). Thus, we can roughly define food allergy as an immune-mediated adverse reaction to food. This immune response can be classified into IgE-mediated, non-IgE-mediated or a mixture of both (Muraro et al., 2014) (**Figure 1**). While IgE-mediated food allergy are responsible for most food allergic reactions and is characterized by the presence of food-specific serum IgE antibody to a food allergen, non-IgE mediated food reactions are associated with cell-mediated mechanisms or antigen-specific antibodies other than IgE (Valenta et al., 2015).

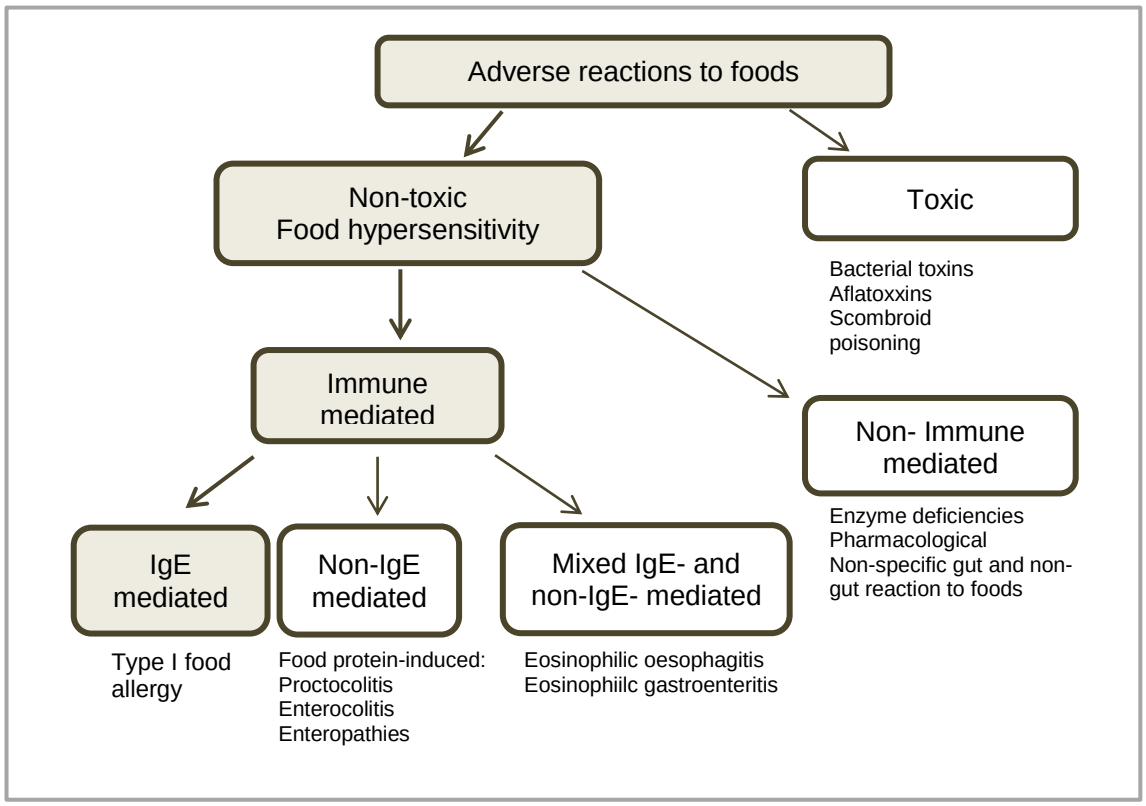

Figure 1. Overview of adverse reactions to food.

1.1.2. Dietary proteins – main food allergens

An allergen is defined as the antigenic molecule giving rise to an allergic response and is virtually always proteinaceous in nature. Many factors may contribute to the overall allergenicity of any given protein (Huby et al., 2000; Bannon, 2004). Some, such the presence of epitopes with allergenic potential, may be essential. Others, such us the glycosylation status, resistance to proteolysis, and enzymatic activity, may play a subsidiary but nevertheless critically important role (Benedé et al., 2013). In view of that, and despite none of these factors is unique, a food allergen possesses three distinct molecular properties; the property to bind IgE antibodies, the property to elicit an allergic reaction and the property to sensitize an individual (Aalberse, 2000).

Although more than 170 foods has been identified as triggers of allergic responses, those causing most of the significant allergic reactions include peanut, tree nut, egg, milk, fish, shellfish, wheat and soy (Sicherer and Sampson, 2010), being cow's milk and egg the most common offending foods in children from continental Europe (Nwaru et al., 2014). Additionally, the number of identified incriminating foods continue to increase, which could either be the result of the globalization and thereby the introduction of new food containing potential new allergenic proteins (Van Putten et al., 2006). Since no single characteristic of a dietary protein is sufficient for predicting its allergenic potential, to develop an improved allergy risk assessment strategy for this novel proteins is a priority action for the research community. Advancing in research for factors influence the intrinsic ability of proteins to act as allergens, as digestibility and/or intestinal abortion, identification of allergenic epitopes and effects of the food matrix and processing on allergenicity are needed (Martos et al., 2013; Benedé et al., 2014).

1.1.3. Immunologic basis of IgE mediated food allergy

Aalthough the immunological changes involved in food allergy have not been yet clearly understood, it is known that depends on a complex network of communicating

immune cells, which are the focus of intensive research. The primary immune cell lineages involved in the initiation and progression of the response include dendritic cells (DCs), mast cells, basophils, eosinophils, T cells and B cells.

Allergic sensitization to proteins involves the induction of an IgE response of sufficient magnitude to facilitate the elicitation of an inflammatory reaction following subsequent exposure to the same (or a cross-reactive) allergen. When sensitization occurs via oral route, the main site to exposure to food allergens is the gastrointestinal tract. This means that for food allergens to initiate allergic sensitization, they must first overcome the normal gut barriers, including acidity, digestion, motility, mucin, layers and tight junction of the enterocytes that prevent passage of macromolecules (Rescigno, 2011).

Two phases can be distinguished in the early pathogenesis of the IgE mediated food allergy; a sensitization and an effector phase (López-Expósito et al., 2013) (**Figure 2**). Briefly, a primary contact with the dietary protein where oral tolerance induction fails or is abrogated, leads to initiate the immune mechanisms when allergen activates antigen presenting cells (APC) (DCs are the major APC population involved) and other immune cells to enhance innate signals which instruct the differentiation of naïve CD4+ T cells preferentially into allergen-specific effector Th2 cells. These innate signals may include membrane bound ligands expressed on DCs, such as OX40L and Jagged (Blázquez and Berin, 2008), that interact with their respective receptors on T cells, as well as soluble mediators from other cells, such as IL-4, IL-25, IL-33 or TSLP. This differentiation and clonal expansion of naïve CD4+ T cells into allergen-specific effector Th2 cells producing IL-4 and IL-13 is followed by the induction of antibody class switching in B cells which are primed to become IgE secreting plasma cells. Allergen-specific IgE (sIgE) binds to the high-affinity receptor FcεRI on the surface of mast cells and basophils and, thus, patient's sensitization results. Effector phase occurs upon a subsequent contact with the allergen when cross linking of the FcεRI-bound IgE on sensitized basophils and mast cells activates them and undergoes degranulation and release of mediators responsible for the classical

symptoms of the immediate phase, such as histamine, cytokines and proteases. (Akdis and Akdis, 2015; Cabrera and Urra, 2015).

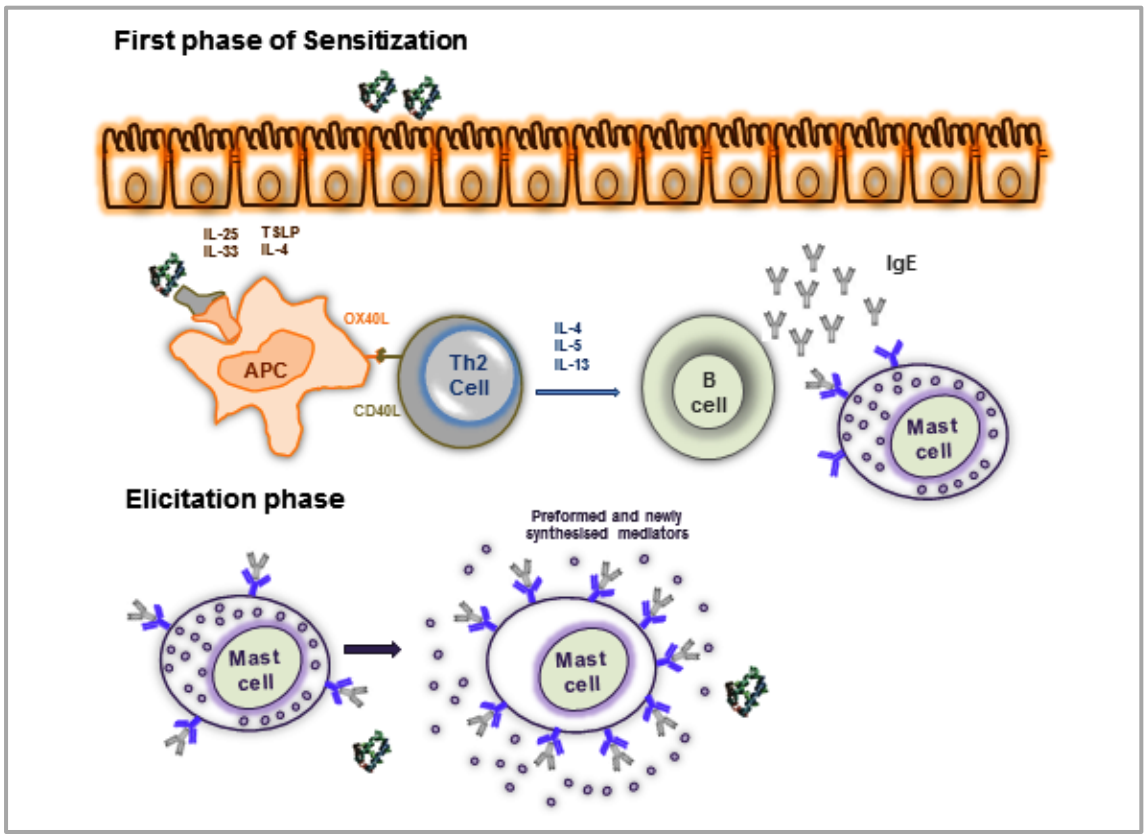

Figure 2. Overview of mechanisms leading to allergic reaction.

1.1.4. Antigen-specific T-cell responses

Naïve T cells are a pluripotent population capable of differentiating into a number of distinct phenotypes upon primary immunization and activation by APC. *Effector T cells*, upon activation in the primary response, secrete cytokines, proteins or peptides that stimulate or interact with other leukocytes, playing a role in orchestrating the immune response to the allergen (Berin and Shreffler, 2008). Some of these primed effector T cells develop into e*ffector memory T cells* (T_{EM}), which upon re-encounter with antigen can rapidly produce a response to confer immediate protection in peripheral tissues. However, according to the duration of antigenic stimulation and the type and amount of cytokines present during priming, some differentiating T cells will become *central memory T cells*

(T_{CM}) with a low activation threshold, which will be maintained for long periods predominantly in lymph nodes, from where they will be ready to expand and differentiate on a future exposure to the same antigen (Sallusto and Lanzavecchia, 2001).

Different signals are necessary for T-cell activation and distinct effector T helper (Th) populations can be defined generally based on their respective cytokine profile. Activated allergen-specific Th2 cells produce IL-4, IL-5, IL-9 and IL-13, considered to drive the allergic response, through the maintenance of allergen-specific IgE (sIgE) levels, eosinophilia, recruitment of inflammatory cells to inflamed tissues, induction smooth muscle contraction and mucus secretion (Akdis and Akdis, 2009). As a consequence of these events, the more severe clinical manifestations of allergy, such as allergic rhinitis, atopic dermatitis, and in extreme cases, systemic anaphylactic reactions appear. Differentiated Th1 cells express high levels of IFN-γ which promote clearance of intracellular pathogens and their presence may counterbalance a dominant Th2 response. In response to allergic stimuli, naïve T cells have also been shown to express high levels of GATA3 and low levels of T-bet, master transcriptional regulators for many Th2 and Th1 genes, respectively (Grogan and Locksley, 2002).

Failure of an immune deviation from an allergen-specific Th2 response to a Th1 immune response has been proposed as the mechanism responsible for allergic disease. However, evidence suggests that dysregulation in the immune system involved in allergy cannot be explained simply by the Th1/Th2 dichotomy, and other effector T cell subsets can contribute to ongoing allergic reactions, such as Th17, Th9 and Th22 (Akdis et al., 2011). Furthermore, in addition to the mentioned effector Th cell subsets, T cells with immunoregulatory properties exist and these are broadly referred as regulatory T (Treg) cells. Treg cells are characterized by the expression of the forkhead transcription factor FoxP3. Functional allergen-specific Treg cells have been demonstrated to have a pivotal role in inducing and maintaining immune tolerance through different mechanisms such as the suppression of mast cells, basophils, and eosinophils; the suppression of inflammatory dendritic cells and induction of tolerogenic dendritic cells; the suppression of allergen-specific Th2 cells, hence preventing the provisional of survival factors for these allergy

effector cells; and the blocking and reduction of IgE production with an early induction of IgG4. All these mechanisms can be mediated via the secretion of IL-10 and/or TGF-β, or through cell contact-dependent suppression. IL-10 and TGF-β suppress IgE production by B cells, and meanwhile, IL-10 induces IgG4. (Palomares et al., 2010; Akdis and Akdis, 2014).

1.1.5. The immune response in the early childhood

Several studies demonstrate that there are great differences, in terms of the characteristics and functionality of the immune system, between children and adults (reviewed in Simon et al., 2015). The immature immune system in early life is rapidly and continuously renewing and producing new cells (mainly T cells developed in the thymus) which make more adaptive responses against a specific antigen than those in adults.

Neonates contain far fewer T cells than adults and it is known that function of early-life T cells is different from adults, reflecting the fetal life, where exposure to foreign antigens is largely restricted (Adkins, 1999). Foreign antigen activation of late fetal or neonatal T cells appear initially skewed towards Th2 immunity which is reinforced by neonatal DCs and epigenetic features (Holt, 2004). Therefore, a maturational deficiency in Th1 function seems to occur, but it tends rapidly to modify during infancy and consolidate presumably through influence of other antigenic exposure. One of the major sites that impacts on the development of the immune system is the gut. Gut environment and bacteria that colonize the gut have a profound influence on the response to many possible antigens (Rescigno, 2014). Other different feature in the newborn immune system is the ability to form and expand new immunological memory comprising memory T and B cells, which becomes increasingly important as it have not yet encountered and established a memory bank to many pathogens (Simon et al., 2015).

This immature scenario might be of special relevance in Treg cells which play a key role immune homeostasis of neonates (Correa-Rocha and Muñoz-Fernández, 2011).

Giving their suppressive nature, Treg cells are crucial in maintaining maternal-fetal tolerance and their values are increased during the pregnancy (Kahn and Baltimore, 2010). These values persist for an extended period of time giving the early-life immune response an anti-inflammatory profile. A dysfunction in Treg subset, either as a consequence of the immune system's immaturity in childhood or by alterations in the generation of the repertoire of Treg, could be implicated in certain pathologies that occur in childhood as allergies, autoimmune diseases or immunodeficiency (Correa-Rocha et al, 2012).

1.1.6. Epidemiology of food allergy

Food allergy constitutes a major public health problem while their prevalence and persistence is increasing throughout the world. Allergic reactions can be life threatening and have far-reaching implications on affected patients and their families, not only upon safety but also on quality of life due to emotional and social restrictions and a remarkable healthcare spending.

On the basis of numerous studies, the true prevalence of food allergy remains unclear because factors such as allergy definitions, study populations, methodologies, geographic variations, ages and dietary exposure, among others, influence the estimates (Sicherer, 2011). More recent estimates indicate that food allergy affects 3.5%–5% of adults and 5%–8% of children in Western countries (Chafen et al., 2010; Nwaru et al., 2014; Sicherer and Sampson, 2014). Although, knowledge about the epidemiology of food allergy is limited and there are global variations, evidence highlights that the prevalence of food allergy is rising in developed countries, with cow's milk, egg and peanut allergy representing most of the burden (Boyce et al., 2010).

1.1.7. Causes of food allergy onset

The causes of food allergy are still unknown and a plethora of risk factors are proposed to influence their development, including race, genetics, dietary and

environmental factors and characteristic of food allergens (Cochrane et al., 2009; Lack, 2012). Besides these questions, it is generally assumed that the increase in prevalence of allergic reactions concur with aspects of the westernized lifestyle such as changes in air pollution, indoor exposure to allergens and a lack of early childhood exposure to infectious agents. Such theory, collectively known as the 'hygiene hypothesis', argues that extreme cleanliness and a lack of early childhood exposure to pathogens, symbiotic harmless organisms in the gut, skin and elsewhere and parasites increases susceptibility to allergic diseases by suppressing natural development of the immune system. It means that children need contact with the microbial biodiversity from the environment for the proper maturation and development of the immune system (Wills-Karp et al., 2001; Okada et al., 2010).

Genetic predisposition seems to be an important determinant; however, no accurate markers associated with food allergy have been identified, suggesting that multiple genes and important gene-environmental interactions have implication for development of food allergy (Hong et al., 2009; Lack, 2012).

The gut microbiota is also likely to be a crucial factor and may help explain why allergy prevalence is increasing. Differences have been found in microbiota between allergic and non-allergic children (Abrahamsson et al., 2012), suggesting certain changes in the pattern of intestinal colonization and microbes may be more important to sensitization than others (Azad et al., 2015; Bunyavanich et al., 2016). Other factors, which may simultaneously influence the gut microbiota, are suggested to contribute for the development of allergy: the age at which solid food is introduced, breast versus formula feeding, degree of gastrointestinal infection, intestinal permeability, mechanisms and site of intestinal antigen, absorption and adjuvant effects (Cochrane et al., 2009).

The marked changes in diet over the past several decades have been also suggested as influential factors; vitamin D insufficiency, reduced consumption of omega-3 fatty acids, reduced consumption of antioxidants and the inflammatory state triggered by obesity (Sicherer and Sampson, 2014). Within this context, vitamin D raises a particular interest since that there is a growing body of literature linking vitamin D status in the regulation of

immune function. More recently variations in vitamin D status and intake have been considered it for epidemiological and immunological studies on allergies (Reinholz, et al, 2012). Nowadays, it is known that several tissues in the body possess receptors for the active form of vitamin D, 1α,25-dihydroxyvitamin D3, and that immune cells including macrophages, epithelial cells and DCs, are capable of converting the circulating inactive form 25-hydroxyvitamin D (25(OH)D) to the active metabolite (Chambers and Hawrylowicz, 2011). This inactive 25(OH)D represents the main circulating vitamin D metabolite and is the most reliable parameter to define human vitamin D status (Heaney, 2012). The active form of vitamin D is an important immune system regulator showing direct effects on naïve and activated Th cells, Treg, activated B cells and DCs (Jones et al., 2012). Between the main immunomodulatory properties of vitamin D, its impact on Treg has been largely proposed. Several studies demonstrated that vitamin D contribute significantly to the induction, survival and preservation of the Treg population (Penna et al., 2005; Chambers and Hawrylowicz, 2011; Vijayendra et al., 2015). Additionally, numerous studies showed a relationship between decreased values of vitamin D, in both mother and infant, and a higher incidence of allergy (Chiu et al., 2015; Jones et al., 2015; Vijayendra et al., 2015). Because of the growing interest, this is a priority area for future research and studies will be crucial to further understand the potential link between vitamin D status and Treg induction and function.

1.1.8. Diagnosis of food allergy

Several diagnostic tools are available for the diagnosis of food allergy (Sicherer and Sampson, 2010; Sampson et al., 2014). First evaluation requires a thorough history and physical examination to determine the possible causal food or food and reaction consistency. To arrive at a diagnosis, specifically whether the food-induced allergic disorder is likely IgE mediated, the clinician should consider appropriate testing that can be evaluated in the context of these prior probability estimates. Serum immunoassays to determine food sIgE antibodies and skin prick tests (SPT) are rapid means to detect

sensitization (Sporik et al., 2000; García-Ara et al., 2001; Heinzerling et al., 2013). However, the appropriate diagnosis became more complicated by the fact that symptoms representative of IgE mediated food allergy may appear in patients without detectable levels of sIgE as well as detection of sIgE does not necessarily correlate with clinical symptoms (Sampson et al., 2014). To confirm diagnosis, an oral food challenge (OFC) is often used, in which the potential allergen is gradually fed in increasing doses under supervision to determine tolerance or clinical reactivity. The gold standard test for the diagnosis of food allergy is the double-blind, placebo-controlled OFC (DBPCFC) as minimizes biases (Bindslev-Jensen et al., 2004). However, there is yet to be universal standardization of the interpretation of challenge results, particularly in research setting. Authorities, the European Academy of Allergology and Clinical Immunology (EAACI) and the American Academy of Asthma, Allergy & Immunology, have published a consensus report on the standardization of DBPCFC (Sampson et al., 2012) and a recent framework for improved documentation and harmonized interpretation of DBPCFCs has been reported (Grabenhenrich et al., 2016).

1.1.9. Treatment of food allergy and future directions

There are no approved interventional treatments for food allergy. The only currently accepted treatment for food allergy is complete avoidance of the offending allergen which can be difficult and has a negative impact on patient and families quality of life.

Considering that, a number of novel therapeutic strategies are under investigation. The therapies undergoing the most extensive research are oral and sublingual immunotherapy, where doses of the dietary protein are given in progressively increasing quantities toward a steady dose, for induction of desensitization. Other food allergen-specific strategies include subcutaneous and epicutaneous immunotherapy (Tordesillas et al., 2017); peptide immunotherapy (Yang et al., 2009; Rupa and Mine, 2012) together the development of peptide-based hypoallergenic derivatives of major food allergens (Lozano-Ojalvo et al.,

2016); and gene therapy using bacterial plasmid DNA. Allergen non-specific strategies include recombinant vaccines; strategies through via antibodies (Berin and Mayer, 2013); the administration of probiotics and prebiotics to manipulate the gut microbiota (Simonyte Sjödin et al., 2016); epigenetics with allergy candidate gene-specific changes in DNA (Hong and Wang, 2014); Chinese herbal medicine called food allergy herbal formula; and the use of anti-cytokines and toll-like receptor agonists (Berin and Sicherer, 2011; Nowak-Wegrzyn and Sampson, 2011; Wood, 2016).

Future challenge is to understand the mechanisms responsible for establishment of food allergy along with restoration of natural or induced tolerance which would enable to do a most accurately diagnosis, prevention, treatment and management of food allergies (Sampson, 2016). In this context, oral immunotherapy attracts considerable attention as successful desensitization has been demonstrated in multiple exploratory trials. However, despite the clear progress and interest toward this therapy, there are still many questions to be answered and parameters to accurately define before OIT becomes an accepted option outside of the research setting (Bégin et al., 2014).

1.2. FOOD ORAL IMMUNOTHERAPY

1.2.1. What is oral immunotherapy?

Oral immunotherapy involves mixing an allergenic food into a vehicle and consuming it in gradually increasing doses (Burbank et al., 2016). Protocols vary in the type of food and vehicle substance used for OIT, whit some using commercially available foods in their natural forms (e. g. liquid milk, ground peanuts) whereas others use prepared products such as defatted peanut flour or dehydrated egg white (EW).

1.2.2. Regimens and phases in OIT

Most food OIT protocols include 2 phases, a first induction phase which is followed by a maintenance phase (**Figure 3**).The induction phase, also called build up phase, often includes an initial escalation stage done over a single day with a fast up-dosing, opening from a very small dose that is gradually increased. If well-tolerated, the dose is escalated incrementally during a buildup period (usually biweekly or weekly) until a target maintenance dose is reached. This phase must be performed in a setting monitored by health professionals, as most reactions occur, and the purpose is to safety begin OIT in a subthreshold starting dose and identify a permissible daily dose for home administration. Generally, the initial doses are in microgram quantities of allergenic protein, often requiring liquid preparation/dilution, and can be advanced to the solid OIT product in the range of several milligrams by the end of this phase. Maintenance therapy continues with daily administration in the home, with a variable duration of months or even years (Nowak-Węgrzyn and Albin, 2015).

A distinction should be made between approaches that induce desensitization versus those in which oral tolerance is achieved. If this maintenance dose is successfully achieved, the patient is said to be desensitized, that is, an increase in the threshold for reactivity is reached but in a desensitized state the protective effect requires continued consumption of the allergenic protein to prevent the reappearance of reactivity. Therefore, after that, the maintenance dose may be discontinued for a pre-specified amount of time and the patient again undergoes a challenge with the offending food. If the subject does not react, he or she has been said to have achieved oral tolerance, which is referred to "sustained unresponsiveness" (Burks et al., 2012; Kobernick and Burks, 2016). This is the ultimate aim of OIT, the ability to tolerate the food after discontinuing ingestion of the allergen for a period of at least 4-12 weeks (Rolinck-Werninghaus et al., 2005). Although a number of studies have demonstrated that the majority of patients treated with OIT can be desensitized successfully to a particular food, sustained unresponsiveness is achieved less commonly (Nowak-Węgrzyn and Albin, 2015). To date, it is unclear whether permanent

tolerance is a function of the duration of OIT and may be achieved in any allergic individual if the OIT is continued long enough or whether some allergic individuals will never become truly tolerant. In view of that, evaluation of immune changes that occur during immunotherapy becomes relevant to gain insight into the mechanisms of allergic sensitization and regulation of tolerance to food allergens.

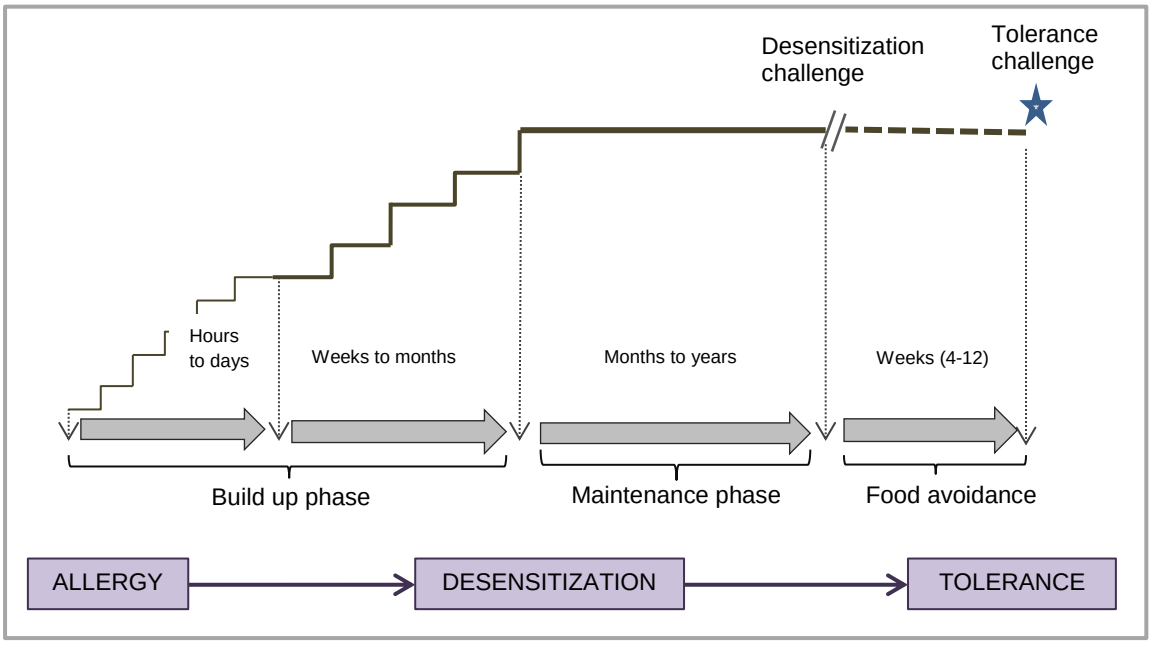

Figure 3. Typical protocol for food oral immunotherapy.

1.2.3. Immune response to food allergens during OIT

There is little current knowledge regarding the immunological changes and mechanisms subjacent to OIT. In humans, the examination of the immunological mechanisms of oral desensitization for food allergy has hampered by several problems. First, unpredictable allergic reactions in patients have resulted in desensitization courses that vary from months to years. In addition, blood samples volumes are small within children and food-specific T cells constitute a very small fraction (< 1%) of peripheral T cell, being difficult to study.

In the early stages, active suppression of immune responses seems to occur with an increase in food-specific IgG4 (sIgG4) (Burks et al., 2012; Vickery et al., 2013) and decreased and mast cell and basophil responsiveness (Thyagarajan et al., 2012). Allergen sIgG4 antibodies are thought to capture the allergen before reaching the effector cell-bound IgE, and thus to prevent the activation of mast cells and basophils and inhibit the release of mediators. Allergen sIgE levels increase initially and then gradually decrease, and some studies have shown that OIT alters the binding pattern of antigen to IgE, possibly through changes in the diversity of epitope recognition or altered antigen affinity (Wang et al., 2010; Vickery et al., 2013). This outcomes agree with a meta-analyses of 21 controlled trials that associate desensitization with a significant reduction in sin prick test responses to the relevant food (mean difference -2.96 mm) and an increase in sIgG4 (mean increase 19.9 µg/ml), whereas do no report a reduction in allergen sIgE (Nurmatov et al., 2014). The evaluation of basophil suppression during OIT has been addressed by several authors. In 2009, *Wanich et al.* showed that allergen-specific basophil reactivity and suppression is associated with clinical unresponsiveness in children with milk allergy. Results of *Thyagarajan et al.* (2012) also demonstrated that OIT suppress basophil responsiveness in a peanut OIT study performing an in vitro stimulation of peripheral blood with peanut allergen and supporting the hypothesis that OIT induced a pathway-specific basophil anergy.

Induction of peripheral T-cell tolerance is a crucial step and the allergen-specific changes in T-cell phenotype during OIT and seems to occurs with a shift away from Th2 cytokine production toward a proinflammatory profile characterized by increased production of IL-1β and TNF-α (Jones et al., 2009; Blumchen et al., 2010; Varshney et al., 2011). However, little information is known about which immune mechanisms or alterations are responsible for triggering this inflammatory cascade that develops the onset of allergy and the evidence about the specific immune subsets implicated in the process of tolerance is very scarce. In a previous study reported by *Fuentes-Aparicio et al.* (2012), it was postulated that OIT highly probable could modify the immune homeostasis and the changes would be reflected in the systemic immune populations. It was observed that

allergic children have a higher percentage and absolute counts of peripheral effector-memory T cells (T_{EM}) than healthy controls, which play a key role in allergy (Tiemessen et al., 2004), and after OIT these values returned to levels similar to those of healthy children (Fuentes-Aparicio et al., 2012). Moreover, interestingly, it was also found that OIT modified values of T cell subsets could being implicated in such origin a marked thymical production of a particular subset of new T cells with a hypo-proliferative and non-reactive phenotype (CD38/ROneg) (Fuentes-Aparicio et al., 2012). The replacement of T_{EM} by a subset of CD38/ROneg cells would ensure a selection of non-reactive T cell clones reducing the immune response against the allergen. A similar observation has been recently reported by Bégin and Nadeau (2015) with data of a peanut OIT study. Authors sequenced T-cell receptors of peanut-proliferative CD4+ T cell, finding a small number of clones consistent overt time only in subjects receiving peanut OIT, which suggest a possible mechanism of replacement of peanut-proliferative T cells. *Ryan et al.* (2016) have also recently investigated this point performing a single-cell sorting and transcriptional profiling of individual T cells collected throughout peanut OIT. Authors showed that sustained OIT success, even after immunotherapy is withdrawn, is associated with the induction, expansion, and maintenance of peanut-specific memory and naive T-cell phenotypes. Thus, an issue of concern in OIT is whether the observed immune changes during the therapy results from a reprogramming of existing allergen-specific T cell clones or from their replacement by different clones to determine the dominant response.

Since the immune response to allergens is the result of a balance between Treg and effector T cells, in the recent years there has been considerable interest about the role of Treg in OIT-mediated desensitization to food. In egg and peanut allergies, OIT protocols have been reported with an in vitro increase of Treg from peripheral blood mononuclear cells (PBMC) (Jones et al., 2009; Varshney et al., 2011; Urra et al., 2012). In a previous egg OIT study, our group also showed the relevance of Treg subset whose frequency and absolute numbers was significant increased when egg desensitization was achieved (Fuentes-Aparicio et al., 2014). Moreover, going further, we observed that OIT induced a profound change in the Treg/T_{EM} ratio, as Treg increase was associated with a decrease in

effector immune cells implicated in the allergic process. Interestingly, data of the same study also allowed seeing a direct correlation between the frequency of effector Treg subset and the number of circulating basophils in egg allergic children.

Presumed mechanisms of action for OIT involved gut immunity, which affect the allergic response through immunomodulation of circulating cells. Oral tolerance is thought to originate in the gut, which support the generation of FoxP3+ Treg cells. There is evidence relating oral tolerance with the capacity of the mucosal DCs to induce FoxP3+ Treg cells in mesenteric lymph nodes. In mouse models, in conjunction with improved tolerance to the food, OIT resulted in an increase in CD4+CD25+FoxP3+ cells and IL-10 and TGF-β-producing Tregs in the lamina propia (Smaldin et al., 2015). The immune responses locally in the gut are poorly understood in human.

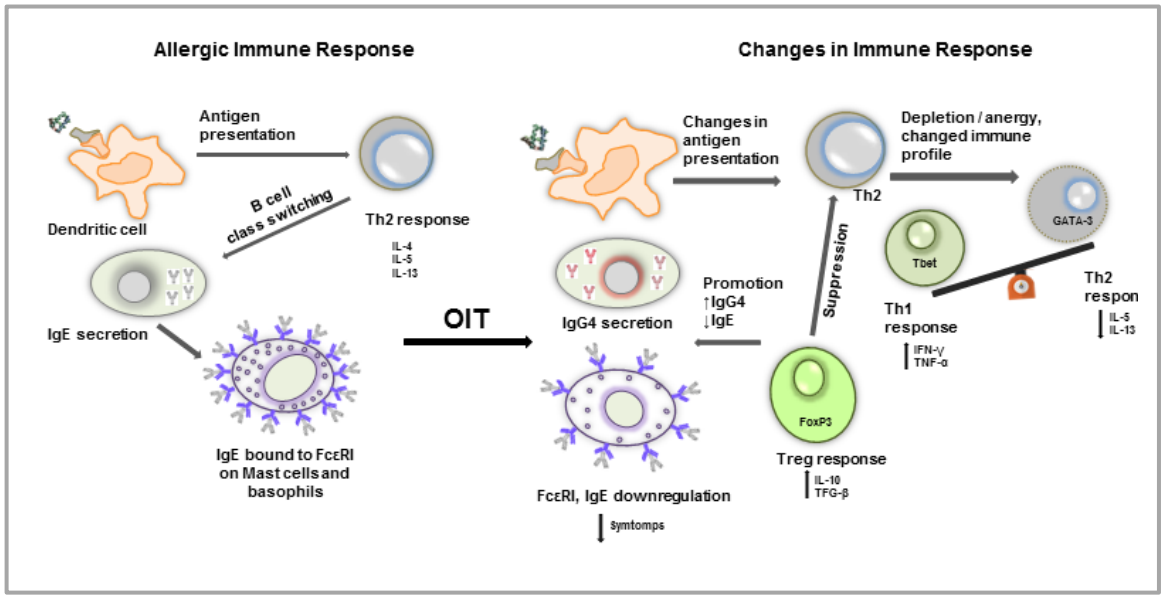

Figure 4. Oral Immunotherapy-induced immune changes in T cells, B cell responses (IgE and IgG4) and basophil activation.

1.2.4. The search for better diagnostic and predictive biomarkers in food allergies

Novel insights into how the immune system works in response to OIT are pointing the way toward development of food allergy biomarkers and therapies.

As mentioned above, particular subsets of CD4+ T cells could constitute a marker of the development of oral tolerance and, therefore, due the potential importance of monitoring T cells during OIT, single-cell approaches and the immune monitoring of individuals undergoing OIT may be useful. Moreover, while peripheral responses of effector Th1 and Th2 cell subsets has been addressed during OIT studies, the involvement of other effector T cell subsets, such as Th9, Th17 and Th22, are poorly investigated. The study reported by *Fuentes-Aparicio et al.* (2012) described a decrease in the serum associated-cytokines to these responses (IL-9, IL-17A and IL-22) of egg allergic children desensitized with egg OIT. In a work of *Dhuban et al.* (2013), Th17 responses to antigen stimulation were impaired in peanut allergic children, suggesting the potential use of IL-17 as biomarker for tolerance to food antigens. However, there is a lack of studies investigating the antigen-specificity of such responses to human OIT.

The possible implication of other allergy-related subsets of cells, as B cells, in successful immunotherapy also raises interest. An aforementioned study of our group (Fuentes-Aparicio et al., 2012) revealed that the percentage of B cells remained unchanged in egg allergic children after OIT. In a recent report of peanut OIT, an early and transient expansion of circulating peanut-specific memory B cells that peaks at week 7 have been described (Patil et al., 2015). Moreover, the kinetics of the induced antigen-specific memory B cell population demonstrates that the rise in peanut-specific IgA, IgG, and IgG4 begins at about the same time as the peak expansion of this population. Thus, one way to address the potential relevance of OIT-induced changes is to isolate antigen-specific B cells and study them on a clonal level. Moreover, the population of regulatory B cells (Breg), which are able to secrete IL-10, are being extensively investigated. The Breg cell subset characterized in human blood as CD24+ CD27+ B cells can negatively regulate monocyte cytokine production via IL-10 dependent pathways (Iwata et al., 2011). Along these preliminary data, B cells and specific Breg subset could be potential biomarkers to study for novel allergen-specific immunotherapies (Fujita et al., 2012).

While it is expected that allergen-specific OIT reflects changes in allergen sIgG4 and sIgE levels, the levels of allergen-specific IgG1 (sIgG1) and IgA are poorly investigated (Savilahti et al., 2014a). IgG4 acts as a blocking antibody for sIgE whereas allergen-specific IgGs inhibit IgE-mediated cell degranulation (Uermösi et al., 2014). Results of a recent trial of egg OIT (Sugimoto et al., 2016) suggests that the presence of high serum levels of allergen sIgG1 after the build-up phase of OIT and high levels of IgA in longer OIT are potentially suitable biomarkers for positive immune responsiveness to OIT.

A recent work has shown that signals from the skin to the gut may govern the perpetuation to allergic reaction to food antigens (Wang, 2016). Epidermal IL-33 and TSLP induced after injury, stress, or environmental factors can trigger the onset of allergic reactions through activation of DCs with the ability to induce a Th2 cell response. In addition, allergic sensitization also results in the increase of intestinal epithelial-derived cytokines TSLP, IL-33 and IL-25, which also propagates the allergic response (Wang and Liu, 2009). IL-33 also promotes the function and maturation of IL-9-producing mucosal mast cells, which increased clinical reactivity to food allergens (Chen et al., 2015; Benedé et al., 2016). Further study of these complex pathways will provide the discovery of biomarkers and therapeutic targets.

Emerging evidence also highlight the important influence of commensal gut microbiota in oral tolerance, as initially suggested by the observation that mice raised in a germ-free environment did not have normal tolerance (Hazebrouck et al., 2009), as well as mice treated with antibiotics or whose gut microbiota were compromised. About human studies, *Bunyavanich et al.* (2016) have recently published an association between early-life gut microbiota and the resolution of cow's milk allergy, suggesting that bacterial taxa within Clostridia and Firmicutes could be studied as probiotic candidates for milk allergy therapy. However, it still remains unclear which bacteria (or other microbes), in which numbers and combinations, and when during the gut colonization process may prevent allergic diseases (Simonyte Sjödin et al., 2016).

1.3. COW'S MILK ORAL IMMUNOTHERAPY

1.3.1. Cow's milk protein allergy overview

Cow's milk protein allergy (CMPA) is the most common food allergy among infants in Europe, with prevalence rates estimated in the range of 2% and 3% (Boyce et al., 2010). Although spontaneous resolution generally occurs in most infants (at around 50%) with IgE-CMA within the first 3-6 years (Elizur et al., 2012; Wood et al., 2013), studies suggest varying results (19-79%) to the rate of resolution during childhood (Skripak et al., 2007; Santos et al., 2010).

Unfortunately, the first line treatment of CMA is the total avoidance of milk to prevent adverse reactions, which can be life-threatening. Nevertheless, milk exclusion involves a wide dietary restriction, which leads to negative nutritional, social, psychological and economic consequences. Moreover, because of cow´s milk (CM) ubiquity, avoidance cannot be always guaranteed and accidental reactions may occur (Boyano-Martínez et al., 2009).

1.3.2. Mayor allergens in cow's milk

Cow's milk contains around 3 to 3.5% of proteins and the main characteristics that should be emphasized are the multiplicity and diversity of proteins that may be involved in allergic sensitization, which include approximately 20 different proteins (Herz, 2008). Moreover, polysensitization to several proteins most often occurs, and the milk proteins of different mammalian species as goat and ewe, become to be potential allergens (Järvinen and Chatchatee, 2009; Rodríguez del Río et al., 2012).

Between cow’s milk protein, caseins (CN, Bos d 8), which constitute 80% of the total milk proteins are described as the most allergenic ones, followed by β-lactoglobulin (β-LG, Bos d 5) and α-lactalbumin (α-LA; Bos d 4), are the major allergens from the remaining whey protein fraction (Monaci et al., 2006). However, proteins present in very low

quantities, such as bovine serum albumin (BSA, Bos d 6), immunoglobulins, and especially lactoferrin, also appear to be important, since some authors have reported even 35%-50% sera from allergic patients respond to those proteins and sometimes to those proteins only (Wal et al., 1995).

The effect of industrial processing (pasteurization, ultra-high-temperature-heating, or dry blending for cow's milk proteins biological activity is minimal (Nowak-Wegrzyn and Fiocchi, 2009). However, in these considerations not only the temperature and time of heating have implications but also the possible interactions within the food matrix could affect (Nowak-Wegrzyn et al., 2008).

1.3.3. Cow's milk Oral Immunotherapy Trials

As in any other food OIT protocol, cow´s milk oral immunotherapy (CM-OIT) involves administering small, increasing doses of cow´s milk during a build up phase followed by a maintenance phase with regular intake of a maximum tolerated amount (around 200 mL) (Brożek et al., 2012; Martorell et al., 2014). Methods and protocols vary, together with patient's characteristic (age, severity or the reactions and level of sensitization), and thus, the results between studies are difficult to compare. In order to put into context how scientific literature on CM-OIT answers some of the most relevant questions on this field, the key features of some of the most relevant CM-OIT studies are summarized below in **Table 1.**, listed in chronological order. In the conventional protocols, the initial build up phase is performed over weeks to months (**Figure 3**). In contrast, an alternative build up phase consists of doses which are doubled and given several times a day over a few days period (commonly 1- to 5-days period) is also reported for cow's milk allergy (Bauer et al., 1999; Martorell et al., 2007; Staden et al., 2008; González-Jimenez et al., 2013), as well as the combination of a rush desensitization followed by a conventional procedure (Longo et al., 2008). This attempt of rush oral immunotherapy (ROIT), tries to be capable to rapidly desensitize patients to allergens, confirming safety and improving the compliance with

therapy. Other additional measures also tries to minimize the rate of adverse reactions, as initiating therapy with sublingual immunotherapy (SLIT) followed by OIT (Keet et al., 2012; Frichmeyer-Guerrerio et al., 2014) or performing the build up phase in combination with anti-IgE monoclonal antibodies (omalizumab) (Nadeau et al., 2011).

Previous studies have shown desensitization rates of 30-92% after a wide array of protocols, but it still has to be established whether a true tolerance with long-lasting effect is achieved and it is possible that the observed very large effects of immunotherapy on achieving tolerance of cow's milk were due to naturally acquired unresponsiveness (Wood et al., 2013). So far, no uniform protocol has been developed and the results are somehow controversial because of the uncertainty of the immunological mechanisms reflected.

Desensitization status, that is, an increasing in the threshold dose which triggers symptoms, has a positive benefit in patient's nutrition and quality of life, allowing patients to eat a wide range of products that contain CM and protecting them against reactions on accidental exposure. However, daily consumption of CM seems to be needed to be maintained and is not known for how long the regular intake must be followed, which is the minimum dose and minimum time interval between single doses (days or weeks) necessary to maintain desensitization and whether the elimination diet may even increase the chance for developing more severe allergic reactions when the ingestion is reintroduced (Niggemann et al., 2006). Regarding this issue, *Pajno et al.* (2013) showed in their CM-OIT trial that the achieved tolerance to CM can be maintained with milk given twice weekly, without a mandatory daily. From today's perspective, CM-OIT has pros and cons and there is still a lack of sufficient evidence for introduced it in the common clinical practice (Yeung et al., 2012; Kostadinova et al., 2013). The disadvantages are the following: risk of and adverse reaction, parent's fear, low compliance, poor accessibility to allergist, and distance to the hospital. In contrast, the benefits observed were dietary improvement and the general subjective perception of a better quality of life.

Before starting the protocol, it is important to thoroughly consider clinical predictors for favorable outcomes in order to identify which patients might represent those for whom

therapy is more appropriated. Children with low to moderate sIgE levels (Staden et al., 2007; Sicherer et al., 2010; García-Ara et al., 2013), low initial starting dose and those who not require adrenaline during induction (Álvaro et al., 2012; Levy et al., 2014) would be expected to respond better to OIT and they are the less likely of spontaneous tolerance (Wood et al., 2013). Nevertheless, these high risk patients would probably be those with the greatest benefit since they are more likely to have persistent food allergy and their reactions can be life threatening.

It remains to be elucidated why some children succeeded in CM-OIT and others do not. Currently, there is little knowledge regarding the immunological changes and mechanisms subjacent to CM-OIT, and therefore, it is crucial to propose predictors to accurately define the efficacy and the risk of adverse reactions. In general, the different studies performed have highlighted an increase in the levels of allergen sIgG4 (Skripak et al., 2008; Pajno et al., 2010; Keet et al., 2012; Savilahti et al., 2014a; Savilahti et al., 2014b; Salmivesi et al., 2016), together with a decrease in allergen sIgE response (Longo et al., 2008; Martorell et al., 2011; Álvaro et al., 2012; Keet et al., 2012; González-Jimenez et al., 2013; Vázquez-Ortiz et al., 2013; Savilahti et al., 2014a; Savilahti et al., 2014b; Salmivesi et al., 2016), although in the case of this sIgE response the results between studies are somehow controversial (Meglio et al., 2004; Zapatero et al., 2008; Pajno et al., 2010; García-Ara et al., 2013). Moreover, CMA resolution involves not only sIgE and Ig4 but also IgG1 and IgA (Savilahti et al., 2014a). In the last years, the diversity and affinity of IgE and IgG4 binding to epitopes on CM proteins have been study in children undergoing CM-OIT and several authors suggest it may help because of such epitope binding correlates with severity of allergic symptoms and the natural development of tolerance in CMA (Savilahti et al., 2014b; Martínez-Botas et al., 2015).

The balance between Treg and promoting Th2 T cells appears to be decisive and whether T cell response and expression of key cytokines are associated with desensitization of milk allergic patients is poorly investigated. To the best of our knowledge, there is only one report available characterizing the CM-specific T-cell response of milk

allergic patients before and after a CM-OIT protocol (Bedoret et al., 2012). Trying to go deeply in this line, *Salmivesi et al.* (2016) have reported the changes in the Th-2 type cytokines IL-4 and IL-5, together with IL-10 involves in Treg response, in the serums of CM allergic patients during a six month CM-OIT intervention. The presumed mechanism of action for CM-OIT through which dendritic cells induce and maintain Th2 allergen-specific cells has been also addressed by *Frichmeyer-Guerrerio et al.* (2014). Authors showed that CM-OIT reduces CM-induced Th2 cytokine responses by CD4+ T cells when co-cultured with plasmacytoid dendritic cells.

Summarizing and taking all of this evidence together, larger clinical studies are needed to verify these findings and a future greater understanding of the immune responses and mechanisms that contribute to the effectiveness of CM-OIT will enhance the efficacy and safety of these therapies made it possible its application as a common CM treatment in the clinical practice.

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Meglio et al. (2004) with follow-up **Meglio et al., (2008)** N=21 Aged 5-10 years	Non-randomized, non-controlled Build up: mean of 201 days, target 200 ml of CM Follow-up: 5 years and 8 months	Desensitization: 71.4% totally desensitized (N=15) 14.3% partially desensitized (40-80 ml) (N=3) Follow up: N=20 70% tolerated CM	<u>Changes OIT between baseline and desensitization:</u> ↓ SPT to CN and α-LA n.s. in CM-sIgE, CN-sIgE and β-LG-sIgE <u>Changes OIT between baseline and long follow up:</u> ↓ CN-sIgE ↓ α-LA-sIgE and ↓ SPT to CN and α-LA <u>Changes between post desensitization and long follow up:</u> ↓ α-LA-sIgE
Martorell et al. (2007) N=4 Aged 19 months- 5 years	**Rush protocol**. Non-randomized, non-controlled Build up: 5 d, target 200 ml of CM Maintenance: 200 ml of CM daily Follow up: 1, 6 months and 1, 2, 3 years	Desensitization: 100% Only 1 case of risk of anaphylaxis solved with medication in few min Long follow up: 100% taking CM with good tolerance	<u>During OIT:</u> ↓ CN-sIgE progressively during longer time of following (not detectable at 3 years)
Staden et al. (2007) N=25 OIT group N=20 Control group Aged 1-3 years	Randomized, controlled Build up: median of 7 months (70 days - 12 months), target 250 ml of CM. Planned in 67 days Maintenance: medium of 9 months (7- 15 months), ≥ 100 ml of CM daily.	Desensitization OIT group: 36% (N=9) full tolerance (250 ml) 12% (N=3) tolerant with regular intake 16% (N=4) partial tolerance Control group: 35% (N=7) tolerant	<u>OIT group vs Control group (baseline):</u> n.s. in sIgE <u>During OIT in OIT group:</u> ↓ sIgE in the group of desensitized children following OIT (N=16) n.s. in sIgE in children who fail desensitization (N=9) <u>During OIT in Control group:</u> ↓ sIgE in the group of children who become tolerant following OIT (N=7) n.s. in sIgE in children who maintain the allergic status (N=13)

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Longo et al. (2008) N= 30 (OIT group) N= 30 (Control group) Aged 5-17 years	**Rush protocol.** Randomized, single-blind, placebo-controlled Build up: rush phase 10 d, target 20 ml of CM with progressive increase at home with target 150 ml Maintenance: ≥ 150 ml CM daily	Desensitization: 30% rush phase in 10 d 0% control Maintenance (12 months): 36% completely and 54% partially (OIT group) 0% Control	<u>OIT group:</u> ↓ CM-sIgE (baseline-6 months (baseline -12 months) <u>Control group:</u> CM-sIgE essentially unchanged in control group, it is only observed a slight reduction in N=3
Skripak et al. (2008) with follow up by **Narisety et al. (2009)** N=13 OIT group N= 7 placebo group with N=6 subsequently open label-treated Aged 6-13 years	Double-blind, placebo-controlled Commercial dry nonfat powdered milk Build up: 10 weeks, target 500 mg (15 ml CM) Maintenance: 500 mg daily If tolerance ≥ 2500 mg (23 weeks) in DBPCFC continue protocol	Desensitization: 92% OIT Median threshold dose with DBPCFC was increased to 5.140 mg compared with 40 mg for placebo Open label-treated group: after OIT the median threshold dose with DBPCFC was increased to 8.140 mg (baseline 40)	<u>During OIT:</u> ↑ CM-sIgG, particularly sIgG4 but suggesting that other IgG subclasses are implicated. (OIT group) ↓ SPT to CM (OIT group) ↓ SPT to CM (Placebo group) n.s.in CM-sIgE <u>Open label-treated group:</u> ↑ CM-sIgG4 and ↓ SPT to CM

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Zapatero et al. (2008) N=18 Aged ≥ 4 years (mean of 5 years)	Non-randomized, non-controlled, prospective Build up: median of 14 weeks (9-32 weeks), target of 250 ml of CM Planned in 10 weeks Maintenance: normal diet with milk drunk freely	Desensitization: 88.8% totally desensitized (N=16) 5.5% partially desensitized (40 ml) (N=1) Follow up: 6 months Long follow up: 100% of totally desensitized children (88.8%) continued a diet without restriction of milk (8 months-1 year)	<u>Changes OIT between baseline and the end of desensitazion:</u> ↓ SPT to CM ↓ CN-sIgE n.s. CM- α-LA- and β-LG sIgE <u>Changes OIT between baseline and 6 mo follow up:</u> ↓ SPT to CM ↓ CN-sIgE n.s. CM- α-LA- and β-LG sIgE
Pajno et al. (2010) N=15 (OIT group) N=15 (Placebo group) Aged 4-10 years	Randomized, singe-blind, Placebo-controlled CM (OIT) Soy milk (placebo) Build up: 18 weeks, target 200 ml Maintenance: 200 ml of CM daily	Desensitization: 67% OIT group 0% Control group Maintenance: 67% (6 months)	<u>OIT vs Placebo:</u> ↑ CM- specific IgG4 (18 weeks) n.s.: CM-specific IgE (transient decrease at early build up) <u>During OIT:</u> ↑ CM-specific IgG4 (baseline-18 weeks) (12 weeks -18 weeks) n.s.: CM-specific IgE

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Martorell et al. (2011) N= 30 OIT group N=30 Control group Aged 2-3 years	Randomized, controlled, parallel-group, multi-center. Build up: 6-12 months, target 200 ml of CM Maintenance: 12-17 months in OIT and 12-15 in Control with ≥ 200 ml CM daily and dairy products without restrictions	Desensitization: 90% OIT group 23% Control group Follow up: 90% following a dairy diet without restrictions (12 months)	<u>OIT from baseline to 12 months:</u> ↓ CM-sIgE ↓ CN-sIgE (OIT group) ↓ SPT to CM (OIT group) n.s. in any markers (Control group) <u>OIT *vs* Control:</u> ↓ CM-sIgE and ↓ CN-sIgE (12 months) ↓ SPT to CM (12 months) n.s. changes at baseline
Nadeau et al. (2011) with immunologic changes in **Bedoret et al. (2012)** N=11 Aged 7-17 years	Non-randomized, non-controlled Dried nonfat powdered CM Build up: 7-11 weeks in combination with omalizumab, target 2 g daily (60 ml CM) Maintenance: 2 g with omalizumab until week 16. Dose-increase to ≥8 g daily (≥240 ml CM) week 24	Desensitization: 81.8 % (7-11 weeks) Maintenance: 81,8% passed DBFC to 8 g and took ≥8 g/d (≥240 ml CM)	<u>OIT (N=5)</u> CM-specific CD4+ T cell proliferation ↓↓1 week from baseline and persisted during build up (8-16 weeks) but ↑ maintenance phase at 3-4 months • IL-10 / TGF-β producing Treg cells not are involved in ↓ CM-specific CD4+ T cell proliferation (10-14 weeks) • Anergy could be involved in ↑ CM-specific CD4+ T cell proliferation after desensitization <u>OIT (N=10)</u> ↑ IFN-γ / IL-4 ratio (weeks 36-52) in fully desensitized patients ↓ CM-sIgE (weeks 36-52) ↑ CM- sIgG4 (weeks >24) ↓ SPT to CM (weeks 52) ↓CM basophil activation markers CD203c+ CD63+ (weeks 24-52)

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Sánchez-García et al. (2011) N=105 Aged 2-15 years	Non-randomized, non-controlled Build up: mean of 19 weeks, target of 200 ml of CM Planned in 16 weeks Maintenance: ≥ 200 ml of CM daily. Other dairy products were allowed except cheese of other species.	Desensitization: 81.9 % (N=86) totally desensitized 19.1% (N=19) failed	<u>Desensitization *vs* failure (baseline):</u> ↓ CM- and CN-sIgE n.s.: SPT to CM; α-LA- and β-LG sIgE NR Changes during CM-OIT
Álvaro et al., (2012) N= 44 allergic anaphylactic during DBPCFC at baseline N=22 allergic non-anaphylactic N=21 negative DBPCFC at baseline (tolerant) Mean age of 8.1 years	Non-randomized, non-controlled Build up: 26.4 weeks in anaphylactic group and 23.1 weeks in non-anaphylactic, target 150 ml of CM Maintenance: ≥ 200 ml daily	Desensitization: Anaphylactic Totally (≥150 ml): 79.5% (N=35) Partially (5-149 ml): 15.9% (N=7) Non-anaphylactic Totally: 72.7% (N=16) Partially: 27.3% (N=6)	<u>Anaphylactic patients vs tolerant (baseline):</u> ↑ CM- and CN-sIgE <u>Anaphylactic patients vs non-anaphylactic (baseline)</u> ↑ CM- and CN-sIgE <u>OIT in anaphylactic patients:</u> ↓ CM- and CN-sIgE <u>OIT in non-anaphylactic patients:</u> ↓ CM- and CN-sIgE

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Keet et al. (2012) N= 10; SLIT N=10; SLIT + OIT(B) N=10; SLIT + OIT(A) Aged 6-21 years	Double-blind, placebo-controlled Aqueous CM extract (SLIT) Powdered CM Build up: SLIT / Median 10 weeks, target 7mg daily SLIT+OIT(B) Median 28 weeks, target 1000 mg daily SLIT+OIT(A) target 2000 mg daily Maintenance: 60 weeks Avoidance: 1 week.	Maintenance: ≥60 weeks 10% SLIT 60% SLIT+ OIT(B) and 80% SLIT + OIT(A) passed OFC (8 g of CM) Tolerance: 10% SLIT 30% SLIT+ OIT(B) and 50% SLIT + OIT(A) passed OFC (8 g of CM)	Outcomes from baseline to 60 weeks (desensitization): In both groups SLIT and SLIT + OIT(A+B) ↑ CM- specific IgG4 ↓ SPT to CM ↓ CD63 CD203c basophil markers Only in SLIT + OIT(A+B) group: ↓ CM-specific IgE ↓ spontaneous histamine release
García-Ara et al. (2013) N= 36 OIT group, subgroups according baseline CM-specific IgE -OIT1 (0.35-3.5 KU/L) -OIT2 (>3.5-17 KU/L) -OIT3 (>17-50 KU/L) N= 19 Control group Aged 4-14 years	Non-randomized, non-controlled Build up: median of 3 months, target 200 ml of CM twice a day Completed build-up in <3 months: 90% OIT1, 50% OIT2, 30% OIT3 Maintenance: 200 ml of CM twice a day and free diet Follow up: 1, 6 and 12 months	Desensitization: 92% (N=33) OIT group (100% OIT1; 88% OIT2; 88% OIT3) Maintenance: 88. 8% (N=32) 20% had experienced adverse events at 6 and 12 months of follow up. Control group: 5.26% (N=1)	OIT from baseline to 6 months of follow up: ↓ Total IgE and CN-sIgE (OIT group) n.s.: CM- α-LA- and β-LG sIgE (OIT group) n.s. in any marker (Control group) OIT from baseline to 12 months of follow up: ↓ Total IgE, CM- and CN-sIgE (OIT group) n.s.: α-LA- and β-LG sIgE (OIT group) ↓CN-sIgE (Control group) Predictors: Children with low or moderate levels of CM-sIgE had a better evolution and less severe symptoms. Children who tasted goat's or sheep's milk cheese had symptoms.

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
González Jiménez et al. (2013) N=18 Aged 3-14 years	**Rush protocol.** Non-randomized, non-controlled multi-center Build up: 5 days, target 200 ml (6.600 mg CM protein) without premedication Maintenance: 200 ml daily.	Desensitization: 39% totally desensitized (200 ml of CM) 61% Partially (mean of 103 ml of CM) Long follow up: 72% consuming CM without restriction (24 months)	<u>During OIT:</u> ↓ CM-sIgE (24 months) ↓ αLa-sIgE (24 months) ↓ CN-sIgE (6, 12, 24 months) n.s.: βLg sIgE
Salmivesi et al. (2013) with changes in biomarkers in **Salmivesi et al. (2016)** N= 18 OIT group N=10 placebo group (subsequently open label-treated) Aged 6-14 years	Double-blind, randomized, placebo-controlled Pasteurized 2.5% fresh milk (OIT) Oat, rice or soy milk (placebo) Build up:162 d, target 6400 mg Maintenance OIT group+ placebo through an open-label OIT, 6400 mg CM protein daily	Desensitization: 89% OIT group Maintenance: 81% consumed CM or milk products 6400 mg CM protein/d (12 months) and 79% (3-3.5 years) Open label-treated group: 100% desensitized	<u>OIT vs Placebo:</u> ↑ serum IL-6 (6 months) ↑ serum IL-10 (6 months) <u>During OIT (N=28 after completed CM-OIT):</u> ↓ Blood eosinophils ↓ serum total IgE ↑ allergen-sIgG ↑ allergen-sIgG4 ↑ serum IL-4 ↑ serum IL-6 ↑ Leptin and resistin (inflammatory adipocytokines)

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Vázquez-Ortiz et al. (2013) N=81 (recruited throughout 5 years) Aged 5-18 years	Non-randomized, non-controlled Build up: 16 weeks, target 200 ml of CM Maintenance: 200 ml daily (median 25 months)	Desensitization: (25 mo) 71.6% totally desensitized (200 ml of CM) Partially desensitized 20.9% (mean of 103 ml of CM)	<u>OIT from baseline to 24months:</u> ↓ CM-sIgE ↓ SPT to CM <u>Predictors:</u> CM-sIgE ≥50 KU/l and SPT ≥ 9 mm at baseline are related with frequent and persistent adverse reactions during CM-OIT
Frichmeyer-Guerrerio et al. (2014) N=8; SLIT N=8; SLIT + OIT(A) N=8 SLIT + OIT(B) Aged 6-17 years	Open label, randomized Aqueous CM extract (SLIT) Powdered CM (OIT) Build up: SLIT median of 10 weeks, target 7mg daily <u>SLIT+OIT(B)</u> Median of 28 weeks, target 1000 mg daily <u>SLIT+OIT(A)</u> target 2000 mg daily Maintenance: ≥60 weeks 1 week avoidance + OFC after 5 weeks (6 months total)	Maintenance: ≥60 weeks 0% SLIT passed OFC 62.5% SLIT+ OIT passed OFC (8 g of CM) Tolerance after avoidance: 31.25% SLIT+ OIT (6 months) (N=5)	<u>Treatment of pDCs and mDCs with CM extract:</u> not induce significant IL-6 or TNF-α, IL-10 (very lows levels of IL-6 and not detectable IL-10) OIT impacts primary on pDCs whereas SLIT affects mDCs <u>TLR responses in DC during OIT between desensitized and tolerant (baseline- 6 months):</u> ↓TLR9-induced pDCs IL-6 responses (both groups) ↓ TLR2- and ↓TLR7/8 induced mDCs IL-6 responses (tolerant) <u>TLR responses in DC-Tcell co-cultures during OIT between SLIT and SLIT+OIT (baseline- 6 months):</u> ↓TLR7-induced pDC-T IL-13 responses (OIT group) ↓TLR7/8-induced mDC-T IL-13 responses (OIT group) ↓TLR7-induced pDC-T IL-10 responses (OIT group) <u>Cytokine secretion in DC-Tcell co-cultures in response to CM:</u> ↓ IL-5 and IL-13 by CD4+ T cells in pDC-Tcell (OIT) n.s. in IFN-γ and IL-10

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Savilahti et al. (2014a) N= 40 Aged 6-17	Non-randomized, non-controlled Build up: median of 191 days, target ≥ 200 ml of CM Maintenance: 200 ml daily	Desensitization: 80% success OIT 20% discontinued OIT Maintenance: 95% consuming CM but 25% with occasional adverse reactions	<u>Predictors of failure at baseline:</u> ↑ CM- sIgE, sIgA,s IgG, sIgG1, sIgG4 <u>Children who success OIT</u> ↑ CM- sIgA, sIgG, sIgG1, sIgG4 ↓ CM-sIgE <u>Children who discontinued OIT</u> ↑ CM- sIgG, sIgG1, sIgG4 n.s.: CM- sIgE, sIgA
Savilahti et al. (2014b) N= 32 OIT group Aged 6-17 years	Non-randomized, non-controlled Build up: median of 186 days (range 167–458) , target 200 ml of CM daily Maintenance: 200 ml daily with follow-up at 3 months	Desensitization: 81% success OIT 19% discontinued OIT (failure)	<u>Success *vs* Failure:</u> ↓ CM-sIgE (baseline and final OIT) ↑ CM-sIgG4 (baseline) <u>During OIT:</u> ↓ CM-sIgE and ↑ CM-sIgG4 ↓ IgE binding to CM epitopes and ↑ IgG4 binding <u>Predictors:</u> ↑Overlap in IgE and IgG4 binding to CM peptides. It is relevant to favorable outcome the capacity of IgG4 to bind the same epitopes that IgE. Children who discontinued OIT had IgE and IgG4 antibodies that bound to CM peptides with greater intensity, broader diversity and greater affinity than in children who successfully completed OIT ↑ sIgE/sIgG4 ratio in CM peptide binding predictor of failure

CM-OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Martinez-Botas et al. (2015) N= 25 OIT group N=7 Control group Aged 4–7 years	Non-randomized, controlled Build up: median of 8 weeks, target 200 ml of CM Maintenance: 24 months with ≤ 200 ml of CM daily on a free diet Follow up: 6, 12, 24 months	Desensitization: 32% ≤ 8 weeks 48% > 8 weeks and > 7 allergic reactions or premedication 20% >7 allergic reactions + premedication Maintenance: 100%	Good correlation between sIgE and sIgG4 binding peptides of five major CM proteins and clinical outcomes. (αS1-CN, αS2-CN, β-CN, κ-CN, β-LG) Number and intensity of IgG4-binding peptides increased continuously during 24 months CM-OIT. Slow and continuous decrease in number and intensity of IgE binding peptides with statistical significance at 24 mo. Authors identified two sets of 16 IgE-binding peptides at baseline suggested as predictors. Early age of starting CM-OIT may be an important factor in the achievement of oral desensitization.

Table 1. Summary of some of the most relevant trials on cow's milk oral immunotherapy.

Abbreviations: DBPCFC, double-blind placebo-controlled food challenge; α-LA, α-Lactalbumin; β-LG, β-Lactoglobulin; CM, cow's milk; CN, casein; n.s., no significant change; mDCS, myeloid dendritic cells; NR, not reported; OFC, oral food challenge; pDCs, plasmacytoid dendritic cells; SLIT, sublingual immunotherapy; sIgE, specific-IgE; sIgG1, specific IgG1; sIgG2, specific IgG2; sIgG4, specific IgG4; SPT, skin prick test

1.4. EGG ORAL IMMUNOTHERAPY

1.4.1. Egg allergy overview

Egg, typically represented in our setting by hen's egg, is considered a substantial food while constitutes a primary protein source in our diet and it is included in a wide range of cooked and manufactured foods. After cow's milk allergy, egg allergy becomes the second most common form of food allergy in pediatric patients in Europe, especially in the first years of life, affecting approximately 1-3% of children (Nwaru et al., 2014; Xepapadaki et al., 2016). In Spain, egg allergy is the most frequent food allergy in young children, with a reported incidence of 2.4-2.6 % in the first 2 years of life (Martorell et al., 2013). Most of the egg allergy reactions show specific IgE positivity against egg proteins and clinical symptoms could compromise the patient's life, having into account that the lowest observed egg protein dose capable of eliciting a reaction may be as low as 2 µg (Morriset et al., 2003). In fact, egg allergy is one of the most common causes of severe anaphylaxis (Caubet and Wang, 2011). Although egg allergy is associated with a high rate of natural tolerance with resolving within the first 6 years of life, the development of this process is not well understood and egg allergy persist in about 50% of children at this age (Sicherer et al., 2014), suggesting the increasing of the clinical disorder in the adulthood.

As other food allergies, the standard treatment of egg allergy is based on the avoidance of egg protein intake. However, due to the broad presence of egg derived components in cooked or manufactured food products, maintaining a strict egg avoidance diet is not easy and dietary failures are relatively frequent, leading to a substantial decrease in the quality of life of both patients and their families (Boyano-Martínez et al., 2012). To overcome the inadvertent transgressions, together with the risks of nutritional deficiencies derived from not consuming egg proteins, in the last few years there has been an increasing numbers of studies focused in the treatment of egg allergy.

1.4.2. Mayor allergens in egg

Most of the allergenic egg proteins are found in EW. Four proteins, ovomucoid (OM) (11%), ovalbumin (OVA) (55%), ovotransferrin (12%) and lysozyme (LZ) (3%), named from Gal d 1 to Gal d 4, respectively, have been identified as the major ones. Although OVA is the most abundant protein comprising EW, OM has shown the highest allergenic activity in egg (OM, OVA LZ in this regard) (Benhamou et al., 2010).

In addition, other minor proteins such as ovomucin, ovoflavoprotein, avidin and ovoinhibitor have also been identified. The yolk has various proteins such as apovitelines, phosvitins and livetins which may also be allergenic, although to a lesser extent (Mine and Yang, 2008).

1.4.3. Egg Oral Immunotherapy Trials

As a promising strategy for treating egg allergy, the aim of egg OIT is to induce a permanent tolerance or alternative desensitization, in order to allow patients to eat this food (or small, hidden doses) without risk of allergic reaction. Based on positive results, egg OIT has been actively researched and a growing number of studies have been performed, especially in the last 10 years. However, as occurs in cow's milk OIT, there are many methodological differences in published studies which prevent us from drawing robust conclusion, such as the study design, the number of patients included, the marked variability between protocols, the extracts used, the time when response treatment is assessed, the varying definition of success, the long-term maintained effectiveness, etc. (Bégin et al., 2014; Praticò et al., 2014; Ibánez et al., 2015; Peters et al., 2016). The key characteristics of some of the most relevant egg OIT studies, including the immunological outcomes and the latter follows-up reported, could be found at the end of this section, summarized in chronological order in **Table 2**.

OIT protocols are performed in IgE-sensitized patients, with history of allergic reactions after ingestion of egg proteins and in most part of the cases with a positive DBPCFC. Although the treatment can be administered at any age, even during the first years of life, most studies tend to be performed in patients older than 6 years with less chance of outgrowing their egg allergy (Sicherer and Sampson, 2014). It has special interest the duration of the build-up phase which range from 1 day to more than 300 days, with different schedules, amount of egg, maximum preprogramed dosage and material used (whole egg, EW; raw, pasteurized, dehydrated, undercooked, hard-boiled or foods that contain egg). Such differences among protocols, equivalences between the doses administered and variances owing to the allergenic potential of the material employed make interpretation and comparison between several egg OIT protocols very difficult.

Maintenance phase usually lasts from 6 to 12 months, but it can be prolonged for years and in some cases it is not defined. The maintenance dose is usually the target dose for the build-up phase and most protocols establish the amount equivalent to 1 egg. The administration interval also varies from once daily to every 2-3 days depending on the previously established target. Patients maintain desensitization only if they consume eggs regularly during this phase, therefore in many studies patients are lost to follow-up owing to poor adherence. Moreover, whether patients maintain this unresponsiveness by following an egg exclusion diet is the relevant question concerned OIT. It is the ultimate goal and few studies have analysed successfully reintroduction of egg into the diet after a period of avoidance (Vickery et al., 2010; Burks et al., 2012; Caminiti et al., 2015; Escudero et al., 2015).

Randomized placebo control studies are considered the “gold standard” in egg OIT since can reduce the influence of unknown or baseline variables, together with the possibility that the observed effects of intervention are due to spontaneous development of tolerance or different treatment settings. However, it can lead to ethical issues, hinder design and procedures complications and make difficult the adherence to the protocol for both children and parents. Thus, only a few studies compared OIT to a placebo (Burks et al., 2012; Caminiti et al., 2015), besides neither were randomized nor controlled (Vickery et

al., 2010; Itoh et al., 2010; García Rodríguez et al., 2011; Sugimoto et al., 2016). Although limited, rush desensitization protocols over days have been also reported. The most relevant reports performed a rapid desensitization schedule with a several daily dose increases include those published by *Itoh et al.* (2010) and *García Rodríguez et al.* (2011).

The immunological mechanisms involved in the clinical changes observed during egg OIT are not entirely clear and data reported by egg OIT studies are limited, while contradictory. The majority of studies report allergen sIgE and IgG4 serum antibody changes and basophils and mast cell responses by increased positivity on skin prick testing. A decrease in the dimensions of the wheal produced by skin prick testing with EW is commonly observed (Vickery et al., 2010; Burks et al., 2012; Dello Iacono et al., 2013; Fuentes-Aparicio et al. 2012; Escudero et al., 2015). It is also generally accepted that levels of egg sIgG4, OVA sIgG4 and EW sIgG4 increase over the time and at early stages (Itoh et al. 2010; Vickery et al., 2010; Escudero et al.; 2015; Sugimoto et al., 2016) even some authors point at egg sIgG4 as a predictor of desensitization (Caminiti et al., 2015). Nevertheless, differences in desensitized children when comparing baseline EW sIgE and egg allergen sIgE values with those observed during treatment are not entirely clear. Some OIT protocols display lower allergen sIgE levels at the end of the immunotherapy (Vickery et al., 2010; Dello Iacono et al., 2013; Meglio et al., 2013; Caminiti et al., 2015; Escudero et al., 2015; Sugimoto et al., 2016), also studies with a ROIT protocol (Itoh et al., 2010; García Rodríguez et al., 2011), but, on the contrary, no significant changes in IgE levels during OIT have also been reported (Burks et al., 2012; Fuentes-Aparicio et al., 2012).

Very few studies have addressed the production of Th1, Th2 and/or Treg egg protein-specific cytokines which reflect the allergen-specific changes in T cells during egg OIT. In their OIT study, *Vickery et al.* (2010) observed a statistically significant reduction in the egg-specific production of IL-13/IFN-γ ratio from 96 hours-stimulated PBMC at 18 months of treatment, whereas such ratio were increasing as egg OIT was prolonged (18-24 months). Same authors also reported an increase in the egg-specific IL-10 levels at 12 months of egg OIT.

In a previous study of *Fuentes-Aparicio et al.* (2012), a broad panel of cytokines was quantified in serums of 19 children egg allergic children before and after egg OIT. A marked reduction in different Th1 and Th2 cytokines were observed after desensitization achievement (IL-2, IL-5, IFN-γ and TNF-α). In the same way, serum IL-10 levels also suffered a reduction after OIT. It is important to highlight that, although there was no significant difference in IL-13 after treatment, it was the only cytokine showed an increasing tendency. *Meglio et al.* (2013) quantified serum levels of IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ and TNF-α, but without could not demonstrate any significant differences for the cytokines tested at 6 months of their egg OIT study, apart from a slightly significant increase of serum IL-5. *Itoh et al.* (2010) also did not found differences for the serum IL-4 and IFN-γ levels at either 6 months or 12 months of their egg ROIT study, albeit observed that IL-10 significantly decreased and TGF-β1 increased at both times points of the treatment. Thus, the mentioned studies showed contradictory data for secretion of IL-10, since no change (Meglio et al., 2013) until an increase (Vickery et al., 2010) or even a decrease (Itoh et al., 2010; Fuentes-Aparicio et al., 2012).

The changes that OIT could reflect in peripheral blood by the presence of modified values of immune subsets have scarcely been studied to date. We previously described that OIT in egg allergic children modifies values of T cell subsets decreasing the percentage and absolute counts of effector-memory CD4+ T cells (T_{EM}) (Fuentes-Aparicio et al., 2012). T_{EM} cells have immediate effector function and can rapidly produce inflammatory mediators, such as Th2-associated cytokines IL-4 and IL-5 (Sallusto and Lanzavecchia, 2001). In addition, we observed in all desensitized children a marked increase in a particular subset of CD4+ T cells whit a hypo-proliferative and non-reactive phenotype (CD4+ CD38+ CD45RO- HLA-DR- cells). Since these cells are probably newly generated in the thymus, we hypothesized that egg OIT induces changes in the immune homeostasis that increases the replenishment by the thymus of the CD4+ pool with this subset of cells, which ensures the selection of non-reactive clones, acquiring this non-reactive phenotype and reducing the immune responses against egg allergens.

Additionally, in vitro experiments identified CD4+ CD38+ cells as great IL-13-secreting T cell subset (Scalzo-Inguanti and Plebanski, 2011) and it is known that interleukin 13 induces IgG4 production and favors class switching to IgE in B lymphocytes (Punnonen et al., 1993).

Other known marker as FoxP3+ Treg cells has been also investigated by *Urra et al.* (2012). Authors reported that unlike controls, children undergoing the egg desensitization protocol showed significantly increased CD4+ FoxP3+ cells in egg white stimulated cells. In agreement with the authors, a previous study in 19 allergy children following an OIT protocol with egg reported an increase in the frequency and absolute counts of Treg associated with egg desensitization (Fuentes-Aparicio et al., 2014). Moving beyond, different phenotypes of these Treg were measured, finding that the increase in the Treg number was more significant for the effector-Treg subset. Thus, the egg OIT could be favoring the generation of peripheral antigen-specific Treg without increasing the differentiation or activation of effector CD4+ T cells.

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Itoh et al. (2010) N=6 Aged 7-12 years	**Rush protocol.** Non-randomized, non-controlled Powdered egg / scrambled egg Rush build up: median of 12 d (9-18), target 1 scrambled egg (60g, 1 whole egg of medium size) Maintenance: at least 1 heated egg twice/week.	Desensitization: 100% Maintenance: 50% did not tolerated 1 g of powdered egg at 9-12 months 100% keeping M dosage without symptoms after 16-21 months	↓ EW- and OM- sIgE ↑ EW- sIgG4 (0-12 months) ↓ Th1/Th2 ratio (0-6 months) ↓ serum IL-10 ↑ serum TGF-β1 (0-6 months and 0-12 months) n.s.: serum IL-4 and IFN-γ, SPT
Vickery et al. (2010) N=8 Aged 3-13 years	Non-randomized, non-controlled Powdered EW Build up: median of 174 d, target of 300 mg Maintenance: mean of 33.8 mo of OIT with a median dose of 2.4 g/d (300 mg - 3.6 g) 1 months of avoidance	Desensitization: 75% (300 mg) 62.5% tolerated 3.9 g in OFC at 4 months 75% keeping dosage at 33 months Tolerance after avoidance: 75%	↓ SPT to EW ↓ EW-sIgE ↓ OM-sIgE ↑ EW-sIgG4 ↑ OM-sIgG4 (0-final build up) The reduction in IgE production is allergen-specific Egg protein-specific cytokines after stimulation PBMCs ↑ IL-10 (0-12 months) ↑ TGF-β (0-6 months) ↓IL-13/IFN-γ (0-18 months) n.s.: CD24+ CD25+ T cells

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
García Rodríguez et al. (2011) N=23 Aged 5-17 years	**Rush protocol.** Non-randomized, non-controlled Pasteurized raw EW / cooked egg Rush build up: 5 d, target 1 whole cooked egg + 8 ml of raw EW Maintenance: 1 cooked egg (24 h first 3 months; 48 h 3-6 months; 72 h after 6 months)	Build up: 60.9% ≤ 5 d 26% 5-10 d Maintenance: keeping dosage after 6-14 mo. 1 patient became symptomatic owing to poor adherence	↓ SPT ↓ EW- sIgE (0-6 months) ↑ EW- sIgG (0-3 weeks, 0-3 months and 0-6 months) ↑FoxP3+ Treg **(Urra et al, 2012)** Differences in SPT, EW-, and OM-sIgE between patients completed Build-up phase ≤ 5 d and those > 5 d
Burks et al. (2012) N=40 OIT group N= 15 Placebo group (10 months) Aged 5-11 years	Double-blinded, randomized, placebo-controlled Dehydrated EW Build up: 10 months, target 2 g Maintenance: 22 months, up to 2 g/d 4-6 weeks of avoidance diet	Desensitization: 55% OIT vs. 0% placebo (5 g in DBPCFC at 10 months) 75% tolerated 10 g in DBPCFC at 22 months Tolerance after avoidance: 28% (10 g at 24 months) Long follow-up: 25% asymptomatic at 36 months	<u>OIT *vs.* placebo:</u> ↓ SPT ↓ Egg CD63+ basophils ↑ Egg-sIgG4 n.s.: Egg-sIgE <u>Differences between patients OIT were desensitized and those were not:</u> Egg-sIgG4 at 10, 22, 24 months from baseline Egg-sIgE at 22 months <u>Predictors:</u> Egg-sIgG4 predicted desensitization at 10, 22, 24 months from baseline SPT predicted desensitization at 22, 24 months from baseline

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Fuentes-Aparicio et al. (2012) with Treg outcomes in **Fuentes-Aparicio et al. (2014)** N=19 OIT group N= 22 Control group Aged 3-14 years	Controlled Dehydrated egg Build up: mean of 9.7 weeks, target 10 g (1 egg) = Tend Maintenance: follow up a 6 and 12 months. Recommended normal diet	Desensitization: 84.2% (N=16)	<u>Desensitized OIT group (N=16) (T0-Tend)</u> ↓ SPT to EW, OVA an OM ↑ EW sIgG ↓ TNF-α, IFN-γ, IL-2, IL-5, IL-10, IL-9, IL-17A, IL-22 ↑% and absolute counts effector-memory CD4+ T cells ↑% absolute counts CD4+ CD45RA+ CD31+ T cells, recent thymic emigrants (T_{EM}) ↑% absolute counts CD4+ CD38+ CD45RO- T cells ↑% and absolute counts of Treg ↑ absolute counts of T_{EM} Treg ↑↑ counts of Treg/ T_{EM} ratio Positive correlation EW sIgG/ CD4+ CD38+ CD45RO- Negative correlation absolute counts of Treg and % of Treg T_{EM} Positive correlation % of Treg T_{EM} and absolute counts of basophils n.s.: % and absolute counts of CD4+ T cells, CD8+ T cells, B cells, Natural Killer cells, monocytes, basophils, neutrophils, eosinophils Total IgE and EW, OVA, OM sIgE IL-12, IL-4, IL-13, IL-1β <u>OIT *vs.* control (T0):</u> ↑% and absolute counts T_{EM} ↓% and absolute counts CD4+ CD38+ CD45RO- <u>OIT *vs.*control (Tend):</u> n.s.: $T_{EM,}$ CD4+ CD38+ CD45RO-

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Dello Iacono et al. (2013) N=10 OIT group N= 10 Control group Aged 5-11 years	Randomized, controlled, home-based Raw EW Build up: not reported time, target 40 ml (1 small egg approx.) Maintenance: 6 months, 10-40 ml/d	Desensitization: 0% total, 90% partial OIT vs. 0% Control (6 months) Median dose tolerated 20 ml. No patients reached maintenance dosage of 40 ml	<u>OIT group:</u> ↓ EW-sIgE ↓ SPT to EW (0-6 months) <u>Control group:</u> n.s. of any biomarker
Meglio et al. (2013) N=10 OIT group N= 10 Control group Aged 4-14 years	Randomized, controlled, home-based Raw EW Build up: mean of 215 d, target 25 ml (3.1 g of egg proteins) Maintenance: 6 months, raw EW at least 3times/week or foods containing same quantity	Desensitization: 80% OIT vs. 20 % Control (at 6 months)	<u>OIT group:</u> ↓ SPT to EW ↓ OM-sIgE ↑ OVA-sIgE ↑ serum IL-5 n.s.: serum IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α (0-6 months) <u>Control group:</u> n.s. of any biomarker

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Caminiti et al. (2015) N=17 OIT group N= 14 Placebo group (4 months) Aged 4-11 years	Randomized, placebo-controlled Dehydrated EW / Cooked or boiled Eggs Build up: 4 months, target 4g (EW contained in 1 egg) Maintenance: 6 months, 2-3 egg/week 3 months of avoidance diet	Desensitization: 94% OIT vs 0% placebo Tolerance after avoidance: 29% OIT vs 7% placebo	<u>OIT vs. placebo (0-4 months):</u> ↑ Egg- sIgG4 n.s.: SPT to egg and sIgE <u>OIT group:</u> ↓ SPT to egg (0-4 months and 0-10 months) ↑ Egg- sIgG4 (0-4 months and 0-10 months) ↓ Egg- sIgE (0-10 months) ↑ SPT to egg ↓ Egg- sIgG4 and ↑ Egg- sIgE patients who lost tolerance after maintenance + avoidance <u>Control group:</u> n.s. of any biomarkers <u>Predictors:</u> <u>Egg-sIgG4 predicted desensitization (14 months)</u>
Escudero et al. (2015) N=30 OIT group N= 31 Control group Aged 5-17 years	Randomized, controlled Dehydrated EW /Undercooked egg (fried, scrambled or omelette) Build up: median of 32.5 d, target 2.8 g Maintenance: 3 months, 1 undercooked egg/48 hours 1 months of avoidance diet	Desensitization: 93% OIT Tolerance after avoidance: 37% OIT vs 3% placebo Long follow-up: 37% asymptomatic at 36 months	<u>OIT vs. placebo (0-4 months):</u> ↓ SPT to OVA and OM ↑ OVA-sIgG4 <u>OIT group (0-4 months):</u> ↓ SPT to EW, OVA and OM ↓ OVA-sIgE and ↑ OVA-sIgG4 ↓ EW-, OVA-, OM-sIgE (3 months) between patients who passed DBPCFC 4 months and those were not <u>Predictors:</u> EW- and OM-sIgE predict the DBPCFC result at 4 months

EGG OIT STUDIES			
Study / Subjects	**Design, material and intervention**	**Efficacy**	**Immunological outcomes**
Sugimoto et al. (2016) N=26 Aged >5 years	Non-randomized, non-controlled Hard-boiled egg Build up: mean of 17 d (8-38) target 1 hard-boiled egg Maintenance: 1 hard-boiled egg/24 hours	Desensitization: 100% Maintenance: 80.8% keeping dosage after 1 year 2 groups: high desensitization (HD) and low desensitization (LD)	↓EW-, OVA- , and OM- sIgE (0-3 months, 0-6 months and 0-12 months) Fast ↑EW-sIgG1, sIgG2, sIgG4 and sIgA (0-1d) and remain at 12mo HD vs. LD (at baseline): ↑EW-, OVA- sIgA ↓OM- sIgG2 HD vs. LD (OIT): ↑EW-sIgA, OVA-sIgA (0-3 months, 0-6 months and 0-12 months) ↑EW-sIgG1 (0-1d) ↑EW-sIgG1 at 1d could be a useful biomarker

Table 2. Summary of some of the most relevant trials on egg oral immunotherapy.

Abbreviations: d, days; DBPCFC, double-blind placebo-controlled food challenge; EW, egg white; n.s.:, no significant change; OFC, oral food challenge; OM, ovomucoid; sIgE, specific-IgE; sIgG1, specific IgG1; sIgG2, specific IgG2; sIgG4, specific IgG4; SPT, skin prick test

2. OBJECTIVES

Food allergy, defined as an immune-mediated adverse reaction to food, is an important health concern since constitutes a potentially life-threatening condition and its prevalence and persistence are undergoing an increase in the last years. Cow's milk is one of the first foods introduced into an infant's diet and, accordingly, is one of the first and most common causes of food allergy in early childhood. Cow's milk protein allergy (CMPA) is associated with a high rate of natural tolerance, disappearing within the first years of life. However, this allergy often precedes the development of other IgE-mediated food allergies, such as the allergy to egg proteins which, similarly, is one of the most frequent food allergies in pediatric patients in Europe. The reason why CMPA appears in some children and not in others, and which are the immune alterations responsible of their establishment in infants are not well understood. Immune system of children significantly differs to those in adults, since it is largely still developing and hence immature, but also has a great plasticity in response to antigens or therapies. Evidence on cow's milk allergy and its appearance would be relevant for the better understating and control of food allergies in the infancy.

Notwithstanding all efforts, there is still no suitable therapy available against food allergy except avoidance, which involves a wide dietary restriction, with negative nutritional, social, psychological and economic consequences. Oral immunotherapy (OIT), which involves giving regular, gradually increased oral dosages of the allergenic protein, is nowadays one of the most studies approaches toward a treatment for food allergy. Although the results are promising, the immune mechanism or alterations subjacent to the process are poorly understood. In the last years many groups have reported on their experience with food OIT, but there are still many methodological differences in published studies which prevent us from drawing robust conclusion, such as the study design, the number of patients included, the allergenic extracts used, the parameters measured to evaluate immune changes, the time when response treatment is assessed, the varying definition of success, the long-term maintained effectiveness, etc.

Under this context, the main purpose of this work is to elucidate the immune mechanisms responsible of the establishment of food allergy in children, why some children

naturally outgrow their food allergy and which are the immune mechanisms by which desensitization occurs in allergic children during an oral immunotherapy treatment.

To this aim, the following secondary concrete objectives are stablished:

- To investigate which immune alterations constitute differential factors between allergy and tolerance, and hence could be implicated in the establishment of cow's milk allergy in infants; elucidating if these crucial factors are good diagnostic or predictive markers, whether they could be behind the establishment of the disorder in the adulthood, and even if they could constitute a good therapeutic target for prevention and/or treatment of cow's milk allergy.

- To investigate which are the immune mechanisms involved in the acquisition of oral desensitization by allergic children during a process of cow's milk or egg oral immunotherapy; identifying biomarkers of tolerance induction, clarifying why some children succeeded in cow's milk and egg oral immunotherapy and others do not; and proposing predictors to accurately define the efficacy and the risk of adverse reactions during cow's milk and egg oral immunotherapy.

3. MATERIALS AND METHODS

3.1. Ethics statements

All human samples and procedures were obtained and performed with written consent from the next of kin, caretakers, or guardians on the behalf of the minors/children involved in the performed studies. All experiments were conducted according to the principles expressed in the Declaration of Helsinki. The Bioethics Committees from the *Consejo Superior de Investigaciones Científicas* (CSIC), Spain, and the *Hospital Universitario La Paz,* Madrid, Spain, approved the studies of OIT performed in the *Hospital Infanta Sofía* of Madrid. Similarly, the Bioethics Committee of the *Hospital Infantil Universitario Niño Jesús*, Madrid, and the *Hospital General Universitario Gregorio Marañón*, Madrid, did the appropriate revision of all procedures within the studies performed in such Hospitals.

3.2. Human peripheral blood samples collection and separation of fractions

Peripheral blood samples were obtained from participants (allergic and non-allergic children) in all performed studies at hospital, at corresponding Division of Allergy or Urgencies, after the informed consent from legal guardians according to ethics statements. The amount of blood was drawn based on minimum requirements and always accordingly to child's age, less than 3 ml of blood in children under 2 years of age and a maximum of 5 ml in older patients.

Blood samples were collected into blood collection tubes treated with ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Samples stored for a no longer period of 8 h were laboratory processed performing methods as described below: human sera were analyzed for total and allergen-specific IgE and IgG4 antibodies and vitamin D testing and human blood cells were used for cell culture and stimulation, flow cytometry analysis and study of gene expression.

Samples from participants in all performed studies were centrifuged at 150g for 15 min at 20ºC to separate sera from the whole blood samples. Sera were placed in cryotubes

stored at -20ºC until serological test for measure total and allergen-specific IgE and IgG4 antibodies were done and a density gradient with Ficoll-Paque Plus media (GE Healthcare, Barcelona, Spain) were used to separate Peripheral Blood Mononuclear Cells (PBMCs). For that procedure, blood was diluted 1:1 with Phosphate Buffered Saline (PBS) without $Ca^{2+/}Mg^{2+}$ and gently layered onto a conical tube with Ficoll. After centrifugation at 500g for 30 min at 20ºC with slow acceleration and without deceleration to prevent mixing of the phases, the layer containing the PBMCs was aspirated from the plasma-Ficoll interface with a disposable transfer pipet, transferred into a new conical tube and washed twice by centrifugation with PBS at 300g for 10 min at 20 ºC. The resulting cell pellet was used for cell-culture or flow cytometry assays.

3.3. Measurement of serum total and allergen-specific IgE and IgG4 antibodies by ImmunoCAP

Levels of total serum IgE and serum allergen-specific IgE and IgG4 were analyzed by ImmunoCAP System (Thermo Fisher Scientific, Waltham, MA, USA). Whole EW (allergen code on CAP platform (f1)), OM (nGal d1 (f233)) and OVA (nGal d2 (f232)) were specific IgE and IgG4 checked in serum of participants in egg OIT studies, whereas whole cow's milk (f2) and total CN (nBos d8 (f78)), α-LA (nBos d4 (f76)) and β-LG (nBos d5 (f77)) were the allergens measured in CM OIT. ImmunoCAP test is designed as a classic "sandwich" immunoassay. Allergen ImmunoCAP covalently bound on a cellulose solid-phase is incubated with 20 µl of serum in the platform. After washing away non-specific IgE or IgG4 bindings (depending on test), enzyme labelled antibodies against IgE or IgG4 are added to form a complex. Following incubation, unbound enzyme-anti-antibody is washed away and the bound complex is then incubated with a developing agent to stop the reaction. The fluorescence of the eluate is measured by the own equipment and the response for the patient samples is transformed to concentrations with the use of a calibration curve. The software analyses and calculates the results.

3.4. Chemiluminiscence assay for vitamin D testing in serum samples

Vitamin D was quantified as 25-hydroxyvitamin D in serum samples to study its potential role in the CMA establishment in infants. Quantification was performed by chemiluminiscence assay at Hospital General Universitario Gregorio Marañón employing the LIASON 25-OH-Vitamin D Total Assay in a LIASON XL analyser (DiaSorin, Stillwater, MN, USA). Values were expressed as ng/mL.

3.5. *In vitro* cell-culture and allergen-specific stimulation of PBMCs

Isolated PBMCs of samples from the Hospital Infanta Sofía were cultured *in vitro* (2×10^6 cells/mL) with OVA or β-CN, egg OIT or cow's milk OIT respectively (both with 200 µg/mL; Sigma-Aldrich, St Louis, MO, USA), in AIM-V medium for 7 days at 37ºC in 5% CO_2. Similar *in vitro* OVA-stimulation of PBMCs were performed in samples from the Hospital Infantil Universitario Niño Jesús but prolonged for only 72 hours. Medium alone was used as negative control and the mitogen phytohemagglutinin (PHA) (4 µg/mL) (Sigma) as a positive control. All experiments were performed in 24-well plates (Corning, Corning, New York) with a final volume of 1ml of medium. After allergen-specific stimulation, the plates were centrifuged at 1100 rpm for 10 min, supernatants frozen at -80ºC until cytokine analysis and cell pellets in Lysis Buffer RA1 (Macherey-Nagel, Duren, Germany) and β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA) or Trizol® reagent (Life Technologies, Carlbad, CA, USA) before RNA isolation.

3.6. Cytokine profile and immune cells subsets by flow cytometry analysis

3.6.1. Cytokine profile analyses by flow cytometric bead array

PBMCs culture supernatants after allergen-specific stimulation were analyzed for the presence of IL-5, IL-13, IL-10, IFN-γ and TNF-α (pg/ml) by using a multiplex flow cytometric bead array (CBA) (BD cytometric bead array; BD Biosciences, San Diego, CA, USA)

according to manufacturer`s instructions. All cytokines were measured under the same conditions and at the same time with 25 µl of culture supernatant, after being captured with the commercial beads conjugated with specific antibodies and the detection reagents, mixture of phycoerythrin (PE)-conjugated antibodies. We designed a Bead Flex Set where sandwiches complexes (capture bead + recognized cytokine + detection reagent) were formed and these complexes were measured by acquiring samples in The Gallios™ flow cytometer (Beckman Coulter, France) and analyzed by Beckman Coulter Kaluza and FCAP Array v3 (BD Biosciences) Software. Each capture bead has a known size and fluorescence, making it possible to detect it using flow cytometry, and the detection reagent provides a fluorescent signal in proportion to the amount of bound cytokine. The cytokines concentrations detected were calculated using 10-points standard curves of each of the human cytokines (0-2500 pg/mL). Results are expressed as the amount of cytokine detected after the stimulation with OVA minus the amount of cytokine detected after *in vitro* stimulation with the negative control.

3.6.2. Percentage of CD4+CD25+Foxp3+ Treg cells in allergen-specific stimulated PBMCs

Percentage of CD4+CD25+Foxp3+ Treg cells was measured after stimulation with OVA for 72 hours of PBMCs from samples of the Hospital Infantil Universitario Niño Jesús. PBMCs were separated, cell surface stained for CD4/CD25 (BD Biosciences) and next fixed and permeabilized with the Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer's instruction, prior to the intracellular staining for transcription factor FoxP3 (BD Biosciences). Isotype controls were included in any antibody staining. Samples were acquired in a Gallios™ flow cytometer (Beckman Coulter, France) and percentages of CD4+CD25+Foxp3+ Treg cells were analyzed by Beckman Coulter Kaluza (BD Biosciences) Software.

3.6.3. Percentage and absolute counts of immune cell subsets in lysed whole blood

The study of the percentage and absolute counts of immune cell subsets could be implicated as biomarkers in CM allergy were performed at the Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain.

The percentage and absolute counts of total T lymphocytes, B lymphocytes and basophils, as well as different subset thereof, were determined by flow cytometry in a Gallios™ flow cytometer (Beckman Coulter, France). We performed two multicolour flow cytometry panels staining a precise volume of 100 µl from whole blood sample for different cell surface markers. After incubation for 30 min in the dark at room temperature, red blood cells were lysed in a TQ-Prep™ Workstation (Beckman Coulter) and, immediately prior to acquisition on the flow cytometer, 100 µl of Flow-Count Fluorospheres (Beckman Coulter) were added to samples in order to determine the absolute numbers of the cell subtypes (cells/µl).

To characterized T lymphocytes, we designed a first flow cytometry panel for which samples were stained with CD3 (T cells), CD4 (CD4+ T cells) and CD8 (CD8+ T cells), including markers for the following subsets: naïve (CD45RA+CD27+), activated (HLA-DR+), central memory (CD45RA−CD27+), effector memory (CD45RA−CD27−). Absolute counts of regulatory T (Treg) cells in total blood were quantified measuring CD3+CD4+CD25+CD127low cells.

Percentage and absolute counts of B cells (CD19+CD3−) were determined by a second flow cytometry panel, including naïve (CD27−IgD+), memory non-switch (CD27+IgD+), memory switch (CD27+IgD−) and regulatory B (Breg) cells (CD24highCD27high) phenotypes. Basophils (CD45lowCD123+IgE+) including activated basophils (CD63+) were also measured.

3.6.4. Percentage of CD4+CD25+Foxp3+Treg and cytokine secreting cells in non-specifically stimulated PBMCs

PBMCs of blood samples from the Hospital Universitario Gregorio Marañón were isolated by density gradient separation using Ficoll-Paque media (GE Healthcare, Barcelona, Spain) as previously described in section 3.3.

A multicolor flow cytometry panel was designed to investigate the percentage of total and subsets of interest of CD4+CD25+Foxp3+ Treg cells. Approximately 10^6 PBMCs were separated and firstly incubated with Human FC block (BD Biosciences, San Diego, CA, USA) for 15 min at 4ºC to block non-specific binding of antibodies to FC receptors. Cell surface marker staining were done for 30 min in the dark at 4ºC for terminally differentiated effector cells (TemRA) (CD45RA+CD27−), and recent thymic emigrants (RTE) (CD45RA+CD27+CD31+) subsets of CD25+Foxp3+ Treg cells. Percentage of Treg cells (CD3+CD4+CD25+Foxp3+) were analysed by Foxp3 intracellular staining with the Anti-Human Foxp3 Staining Set (eBioscience, San Diego, California) according to the manufacturer's instructions. Briefly, after the staining of cell surface antigens, cells were washed, incubated with a fixation/permeabilization solution for 30 min in the dark at 4ºC, washed again twice and stained for intracellular cytokines for another 30 min at 4ºC.

Analysis of cytokine-secreting CD4+ T cells was performed in PBMCs non-specifically stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and Ionomycin (Io) (1 µg/ml) (both from Sigma-Aldrich, St. Louis, MO) for 5h at 37ºC in 5% CO_2 and including the presence of GolgiStop protein transport inhibitor (0.7ul/ml) (BD Biosciences, San Diego, CA, USA). PBMCs were surface stained for 30 min in the dark at 4ºC with markers including activation (CD69+), Th1 response (CXCR3+), Th2 response (CCR4+CCR6-) and Th17 response (CCR4+CCR6+CXCR3-). Viability was assessed by labelling dead cells with fixable viable dye (eBioscience) Intracellular staining of IFN-γ (Th1 response), IL-4 (Th2 response), and IL-17 (Th17 response) was done following the instructions of the Cytofix/Cytoperm Kit (Beckton Dickinson). Frequency of cytokine-secreting cells was calculated in total CD4+ T cells.

3.7. Relative gene expression

3.7.1. Separation and quality assessment of total RNA

Total RNA from *in vitro* cultured and allergen-specific stimulated PBMCs was extracted by using the Total RNA Isolation NucleoSpin® RNA II Kit (Macherey-Nagel, Duren, Germany) in samples from the Hospital Infanta Sofía, or iPrep™ Trizol® Plus RNA Kit (Life Technologies, Carlbad, CA, USA) in samples from the Hospital Infantil Universitario Niño Jesús, according to the manufacturer´s instructions. The RNA template was qualitatively assessed and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and a NanoDrop ND1000 instrument (Thermo Fisher Scientific), respectively.

3.7.2. Quantitative Real-Time Polymerase Chain Reaction

Reverse transcription reactions were performed following the manufacturer's instructions with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Manheim, Germany). RT-qPCR was performed in a ViiA™ 7 Real-Time PCR System (Applied Biosytems) using a total of 6 ng of transcribed cDNA and TaqMan® Gene Expression Assay for the transcription factors: GATA3 (Human Assay ID Hs00231122m1), T-bet (ID Hs00203436m1) and FoxP3 (ID Hs01085834m1), according to manufacturer´s recommendations. The hypoxanthine guanine phosphoribosyl transferase (HPRT) (ID Hs02800695m1) was used as reference gene. The amplification program used was: 1 cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and finally 1 cycle of 1 min at 60 °C. All reactions were performed in triplicate. The mean value of the replicates for each sample was expressed as the quantification cycle (Cq). The relative gene expression values (RQ) of a gene between two times of treatment were calculated as reported by Livak and Schmittgen (2001). A RQ value of sample higher than 2 or lower 0.5 was established to be considered a relevant change (RQ=1 showed no change).

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (San Diego, CA, USA) and SPSS software (IBM, New York, USA). Shapiro-Wilk test was used for assessing normality of variables. Non-parametric Mann–Whitney test was used for comparison a variable of interest between two independent groups of participants, and Wilcoxon test when comparing a variable between to related samples to analyse differences during OIT. Correlation between variables was established by Pearson correlation (PC) test. Capacity of discrimination between groups for the different variables was analyzed by ROC's curves. *P*-value <0.05 by 2-sided test was considered significant.

4. RESULTS AND DISCUSSION

4.1. The establishment of cow's milk protein allergy in infants

This study aimed to investigate the immune alterations which constitute differential factors between allergy and tolerance in pediatric cow's milk protein allergy, and hence could be implicated in its establishment in infants.

The results reported in this section have been published in the reference: [Perezabad, L., López-Abente, J., Alonso-Lebrero, E., Seoane, E., Pion, M., Correa-Rocha, R. (2017). The establishment of cow's milk protein allergy in infants is related with a deficit of regulatory T cells (Treg) and vitamin D. *Pediatric Research*. doi: 10.1038/pr.2017.12].

4.1.1. RESULTS

Participant selection, samples and follow up

Infants younger than 9 months, with symptoms compatible with cow's milk protein allergy (CMPA), such as vomiting or skin eruptions after cow's milk (CM) ingestion were recruited at *Pediatric Allergy Division or Urgencies* of the *Hospital General Universitario Gregorio Marañón* (Madrid, Spain). The confirmation of CMPA diagnosis was performed by Oral Food Challenge (OFC) (unless contraindicated by severe clinical profile), as well as considering the total and CM-specific IgE values, physical examination and family history. Patients with a diagnosis of non-IgE mediated CMPA, cow's milk intolerance, allergic proctocolitis, or enterocolitis, were excluded from the study. Egg allergy, which is also frequent at this age, was discarded in all patients. As follows, after applying the inclusion and exclusion criteria, the children enrolled in the study were distributed in two groups: 1) infants with a confirmed diagnosis of CMPA (CMPA group) by immediate symptoms after OFC (N=15); and 2) age-matched non-allergic controls (Control group) (N=13) with a negative result for the OFC, and subsequent ingestion of milk at home without developing symptoms.

Peripheral blood samples were obtained between 1 and 4 days after the first CM adverse reaction and just before the oral challenge test and the diagnosis of the infants. Samples were processed immediately as described in Material and Methods (section 3.2).

Patients included in the CMPA group were clinically followed along a year to determine whether they became tolerant to milk. A patient was considered tolerant by the absence of allergic reaction after a controlled exposition to milk and confirmed if the patient was able to consume CM in a normal quantity without adverse reactions.

Patient's demographics and specific IgE response

Twenty-eight participants were enrolled, divided into two groups. There were no significant differences in sex and age between groups, being the mean age in Control and CMPA groups 6.18 and 6.43 months, respectively. As expected, total and specific IgE to CM, α-LA, CN and β-LG were increased in the CMPA group but not in the Control group. Values are registered in **Table 3.**

	Control (N= 13)	CMPA (N= 15)	*P*-value
Age (months)	6.18 ± 0.62	6.43 ± 0.46	0.747
Gender (% male)	53.85 % (7/13)	60.0 % (9/15)	0.747
Total IgE	8.90 ± 2.87	65.35 ± 27.63	**0.005***
CM specific IgE	0.10 ± 0.04	4.71 ± 1.77	**0.000***
α-LA specific IgE	0.01 ± 0.01	1.67 ± 0.69	**0.003***
CN specific IgE	0.06 ± 0.03	1,00 ± 0.44	**0.003***
B-LG specific IgE	0.05 ± 0.03	4.36 ± 2.15	**0.000***

Table 3. Demographics and specific IgE response of children included in Control and CMPA groups. Values are presented as mean ± SEM. * *P*-value <0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values. Plasma values of IgE are expressed as kU/L.

Extensive analysis of immune subsets and cytokines

Percentage and absolute counts (cells per μL of total blood) of a wide range of immune cell subsets were compared between children with CMPA and Controls (**Table 4**). Interestingly, there were very few differences between the values observed in CMPA and control children, and percentages and absolute counts for CD4+ and CD8+ T cells, including subsets naïve (CD45RA+CD27+), central memory (CD45RA-CD27+), effector memory (CD45RA-CD27-) and activated (HLADR+); B cells (CD19+CD3-) covering naïve (CD27-IgD+), memory switch (CD27+IgD-), memory non-switch (CD27-IgD+) and Breg (CD24highCD27high); and basophils (CD45lowCD123+IgE+) including activated basophils

(CD63+) were comparable between both groups. Significant differences were only found for naïve CD8 T cell counts, which were lower in CMPA children than in controls.

	Percentage			Cells/µL		
	Control (N= 13)	CMPA (N= 15)	*P-value*	Control (N= 13)	CMPA (N= 15)	*P-value*
Total Lymphocytes						
CD4+ T cells	46.54 ± 2.97	49.81 ± 2.89	0.345	3056 ± 220	2632 ± 212	0.420
CD8+ T cells	12.41 ± 1.16	12.69 ± 1.17	0.534	798 ± 81	642 ± 46	0.123
B cells	24.89 ± 2.18	24.64 ± 1.69	0.872	1669 ± 192	1304 ± 113	0.123
CD4+ T cells						
Naive	82.60 ± 1.75	79.04 ± 3.71	0.884	2530 ± 197	2151 ± 210	0.332
Central Memory	9.37 ± 1.19	10.03 ± 1.07	0.872	279 ± 32	236 ± 16	0.084
Effector Memory	0.56 ± 0.10	1.63 ± 1.08	0.533	17.3 ± 3.6	19.8 ± 5.0	0.982
Activated	1.02 ± 0.13	1.72 ± 0.68	0.927	31.2 ± 3.8	30.6 ± 4.0	0.565
CD8+ T cells						
Naive	84.32 ± 3.54	77.35 ± 6.04	0.497	671 ± 82	477 ± 45	**0.023***
Central Memory	8.73 ± 1.70	12.17 ± 2,38	0.300	67.0 ± 13.5	80.8 ± 20.2	0.596
Effector Memory	1.87 ± 0.98	2.60 ± 1.46	0.712	14.5 ± 7.0	22.2 ± 13.4	0.982
Activated	2.68 ± 0.78	6.58 ± 2.85	0.420	21.4 ± 6.3	48.0 ± 22.9	0.836
B cells						
Naive	91.41 ± 0.91	90.55 ± 0.74	0.357	1534 ± 184	1118 ± 129	0.069
Memory switch	0.99 ± 0.17	1.09 ± 0.14	0.447	15.2 ± 2.2	13.3 ± 1.8	0.534
Memory non-switch	6.09 ± 0.80	6.77 ± 0.66	0.357	95.0 ± 12.3	86.2 ± 9.6	0.662
Breg	1.48 ± 0.17	1.72 ± 0.30	0.765	22.4 ± 2.3	21.2 ± 3.0	0.420
Basophils						
Total	0.60 ± 0.03[a]	0.65 ± 0.08[a]	0.982	58.3 ± 3.5	57.1 ± 7.3	0.475
Activated	40.11 ± 4.73	38.13 ± 4.73	0.695	23.8 ± 3.6	23.0 ± 5.0	0.504

Table 4. Values of percentages and absolute counts (cells per µL of total blood) for immune cell subsets in Control and CMPA children enrolled in the study. Values are given as mean ± SEM. *: *P* <0.05 in non-parametric Mann-Whitney test comparing groups. Percentage for each subset is calculated regarding the total of the corresponding population (Total Lymphocytes; CD4+ T cells; CD8+ T cells; B cells or Basophils). [a] percentage of basophils in total leucocytes (CD45+).

PBMCs from CMPA infants were non-specifically stimulated *in vitro* with PMA and ionomicine, and the percentage of Th1, Th2 and Th17 cytokine-secreting CD4+ T cells

analysed. There were no differences neither in the frequency of IFN-γ secreting CD4+ T cells (Th1) (Mean ± SEM CMPA=3.51 ± 2.09; Control=1.82 ±0.34; *P*=0.742), nor in the frequency of IL17-secreting CD4+ T cells (Th17) (CMPA=0.14 ±0.03; Control=0.10 ± 0.02; *P*=0.727). However, the frequency of IL4-secreting CD4+ T cells (Th2) was significantly higher in CMPA children (**Figure 5**) (CMPA=0.69 ± 0.13; Control=0.43 ± 0.06; *P*=0.037).

Between the different variables studied here, the higher frequency of IL4-secreting CD4+ T cells (IL4-TCD4) seems to be the only differential immune factor in CMPA children that could be associated with the appearance of symptoms and the development of this allergy.

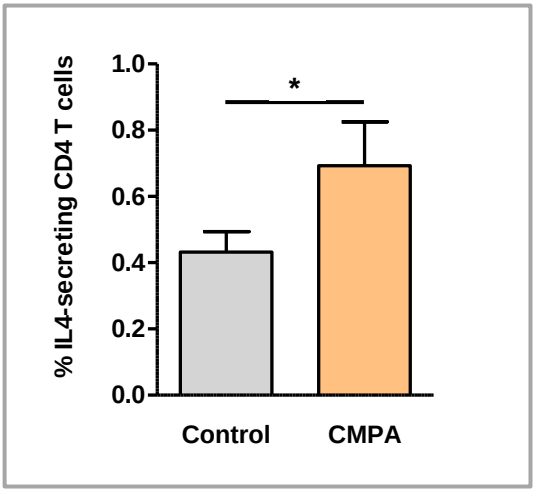

Figure 5. Percentage of IL4-secreting CD4+ T cells measured by intracellular staining in PMA + Io stimulated PBMCs. Median with interquartile range in both Control and CMPA groups are represented. *: *P*<0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values.

Values of regulatory T cells and immune homeostasis

It was expected that increased frequency of (effector) IL4-secreting CD4+ T cells would promote the expansion/activation of Treg cells in CMPA patients. The percentage of Treg cells into the CD4+ T cell population, which is a relative measure that can be influenced by the expansion or depletion of other CD4+ T cell subsets, was comparable between Control and CMPA groups of children (**Figure 6A;** *P*=0.433). However, when

absolute counts of Treg cells (cells per µL of total blood) were measured, significantly lower Treg numbers in the CMPA group were found compared with Controls (**Figure 6B**; *P*=0.040).

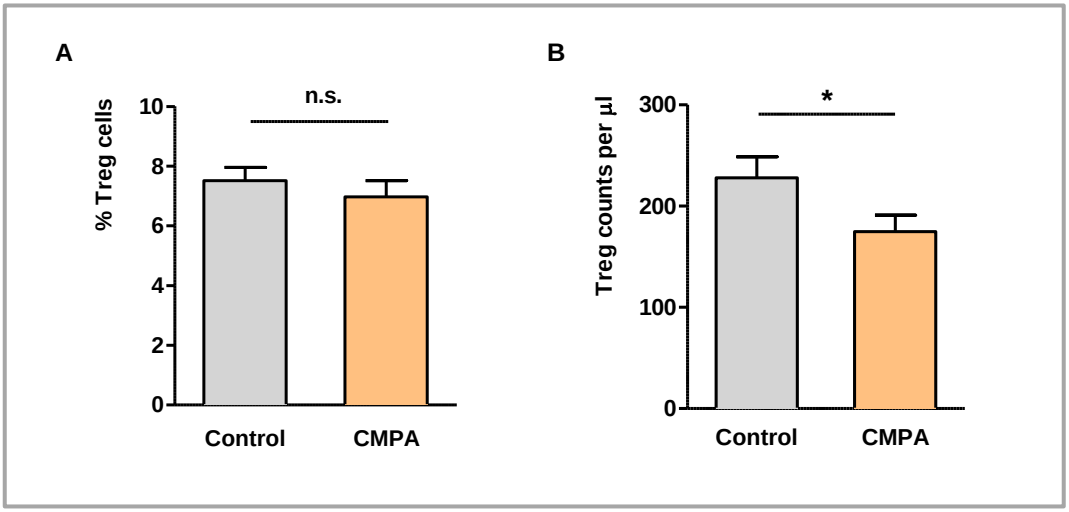

Figure 6. Values of Treg cells. Percentage (**A**) and absolute counts (cells per µL of total blood) (**B**) of Treg cells. Median with interquartile range in both Control and CMPA groups are represented. *: *P*<0.05, n.s.: non-significant differences in non-parametric Mann-Whitney test comparing Control and CMPA values.

After analysing the phenotype and the differentiation stage of these Treg cells, the absolute counts of all the Treg subsets were lower in the CMPA group in comparison to controls (**Table 5**), and this deficiency was significant for the central memory (CD45RA-CD27+) (**Figure 7A**; *P*=0.040) and terminally differentiated effector cells (TemRA) (CD45RA+CD27−) (**Figure 7B**; *P*=0.022) Treg subsets. Additionally, only in the CMPA group, a negative correlation between the frequency of IL4-TCD4 cells and naïve Foxp3+CD25+ Treg cells (PC=−0.783; *P*=0.003) was observed, together with a positive correlation with the frequency of activated Treg (PC=0.881; *P*=0.000). In other words, a high frequency of IL4-TCD4 cells is associated with a decrease in the proportion of naïve Treg and an increase in the proportion of activated Treg, which could reflect an active differentiation of Treg cells from a naïve to an activated phenotype in response to the increased frequency of IL4-TCD4 cells.

	Percentage			Cells/µL		
	Control (N= 13)	CMPA (N= 15)	*P-value*	Control (N= 13)	CMPA (N= 15)	*P-value*
Treg Cells[a]	7.51 ± 0.44	6.97 ± 0.54	0.433	227.9 ± 21.0	174.8 ± 16.4	**0.040***
Naïve[b]	69.73 ± 2.86	64.89 ± 3.67	0.528	157.3 ± 14.3	120.0 ± 13.4	0.081
Central Memory[b]	19.26 ± 2.74	19.52 ± 1.57	0.982	43.12 ± 5.60	32.68 ± 3.99	**0.040***
Effector Memory[b]	1.20 ± 0.35	1.91 ± 0.83	0.311	2.89 ± 0.97	2.38 ± 0.47	0.945
Activated[b]	3.52 ± 0.34	4.10 ± 0.98	0.534	7.95 ± 0.96	6.37 ± 1.34	0.117
TemRA[b]	0.57 ± 0.21	0.09 ± 0.01	**0.027***	1.28 ± 0.45	0.16 ± 0.02	**0.022***

Table 5. Values of percentages and absolute counts (cells per µL of total blood) for Treg and Treg subsets. Values are given as mean ± SEM. *: *P*<0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values. [a] Percentage of Treg cells in the total of CD4+ T cells; [b] Percentage of Treg subsets in the total of Treg cells.

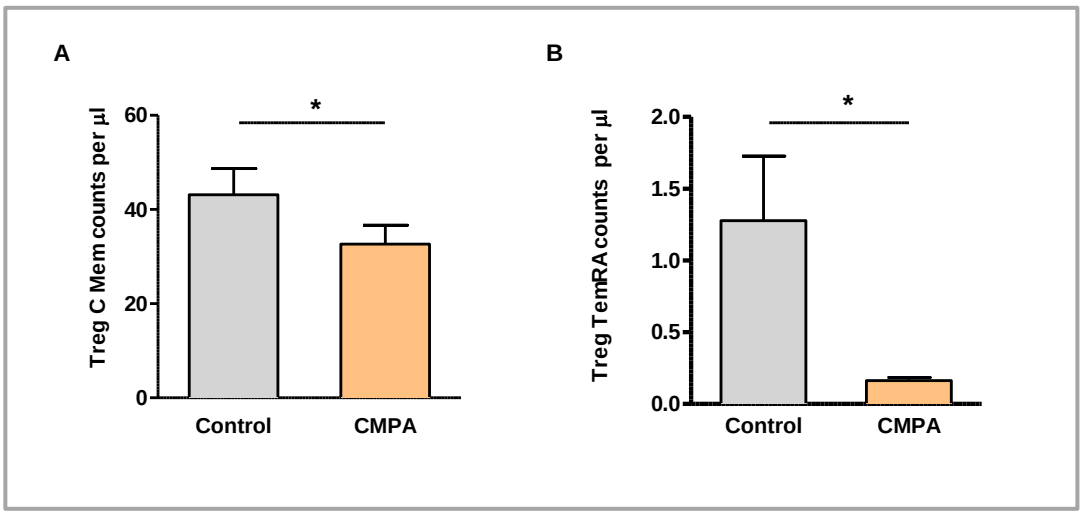

Figure 7. Values of Treg cells. Absolute counts of Central memory (C Mem) Treg cells (**A**) and terminally differentiated effector cells (TemRA) Treg cells (**B**). Median with interquartile range in both Control and CMPA groups are represented. *: *P*<0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values.

We also analysed the ratio between Treg and IL4-TCD4 cells, which is an indicator of the balance between immune tolerance and immune reactivity. The ratio was lower in the CMPA group than controls, reflecting a Treg imbalance in CMPA patients. The differences in the Treg/IL4-TCD4 ratio were significant in both cases, when percentage (*P*=0.036) or

absolute counts (*P*=0.027, **Figure 8**) of Treg and IL4-TCD4 cells were used to calculate the ratio between these subsets. The Treg/IL4-TCD4 imbalance and the deficit in the number of circulating Treg cells in CMPA infants could result in an inadequate control of effector T cells, and could explain the increased frequency of IL4-TCD4 cells. In fact, in the CMPA group but not in controls there was a negative correlation between Treg absolute counts and IL4-TCD4 cells. The lower was the quantity of circulating Treg the higher was the frequency of IL4-TCD4 cells (PC=−0.614; *P*=0.019).

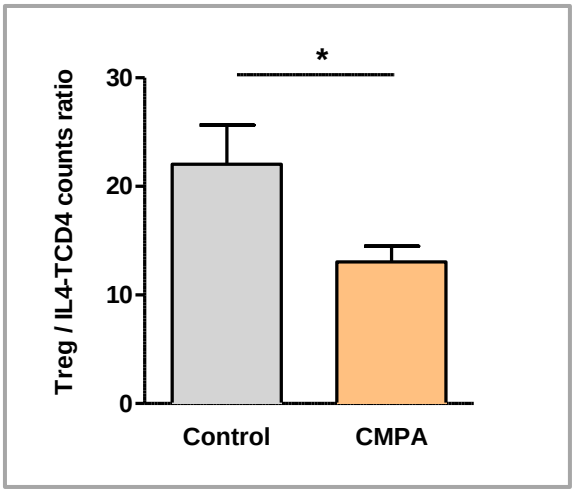

Figure 8. Ratio between Treg and IL4-secreting CD4+ T cells (IL4-TCD4) absolute counts. Median with interquartile range in both Control and CMPA groups are represented. *: *P*<0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values.

Mechanisms of Treg cells deficit. Thymic function and Vitamin D levels

There are different mechanisms that could explain the decreased Treg numbers observed in the CMPA group. Because Treg cells is a subset of CD4+ T cells, which is also generated in the thymus, a deficiency in the thymic production of Treg cells could be related with the reduced number observed in periphery. The expression of CD31 within the pool of Foxp3+CD25+ Treg cells were analysed, a marker which is only expressed in recent thymic emigrants (RTE) and it is considered an indirect indicator of thymic production (Kimmig et al., 2002). The results showed that there are not any significant

differences in the frequency (*P*=0.703) and absolute counts (*P*=0.560) of RTE Treg (CD45RA+CD27+CD31+) between the CMPA and control group (**Figure 9A, 9B**), and hence, a possible defect in the thymic production as the reason of Treg deficiency can be discarded.

Other mechanism that could be implicated in a deficit of Treg cells is the serum levels of 25-hydroxyvitamin D. Values of vitamin D, quantified as 25(OH)D (ng/mL), were significantly lower in the CMPA group than in control children (CMPA=35.3 ± 3.5; Control=47.9 ± 3.7; *P*=0.041) (**Figure 9C**). Moreover, a direct correlation between plasma levels of Vitamin D and absolute counts of Treg were observed (PC=0.390; *P*=0.027). Serum 25(OH)D values lower than 30 ng/mL are considered insufficient in children (Muehleisen and Gallo, 2013). Even if the median values of vitamin D in the CMPA group are higher than this cut-off, we observed that the only four children with values lower than 30 ng/mL all belonged to the CMPA group (**Figure 9D**). In addition, children included in the CMPA group were those with lower vitamin D values and lower Treg counts (orange dots, **Figure 9D**), supporting the hypothesis that the deficiency in vitamin D could be related with the deficit of Treg cells and the subsequent increased Th2 immune responses.

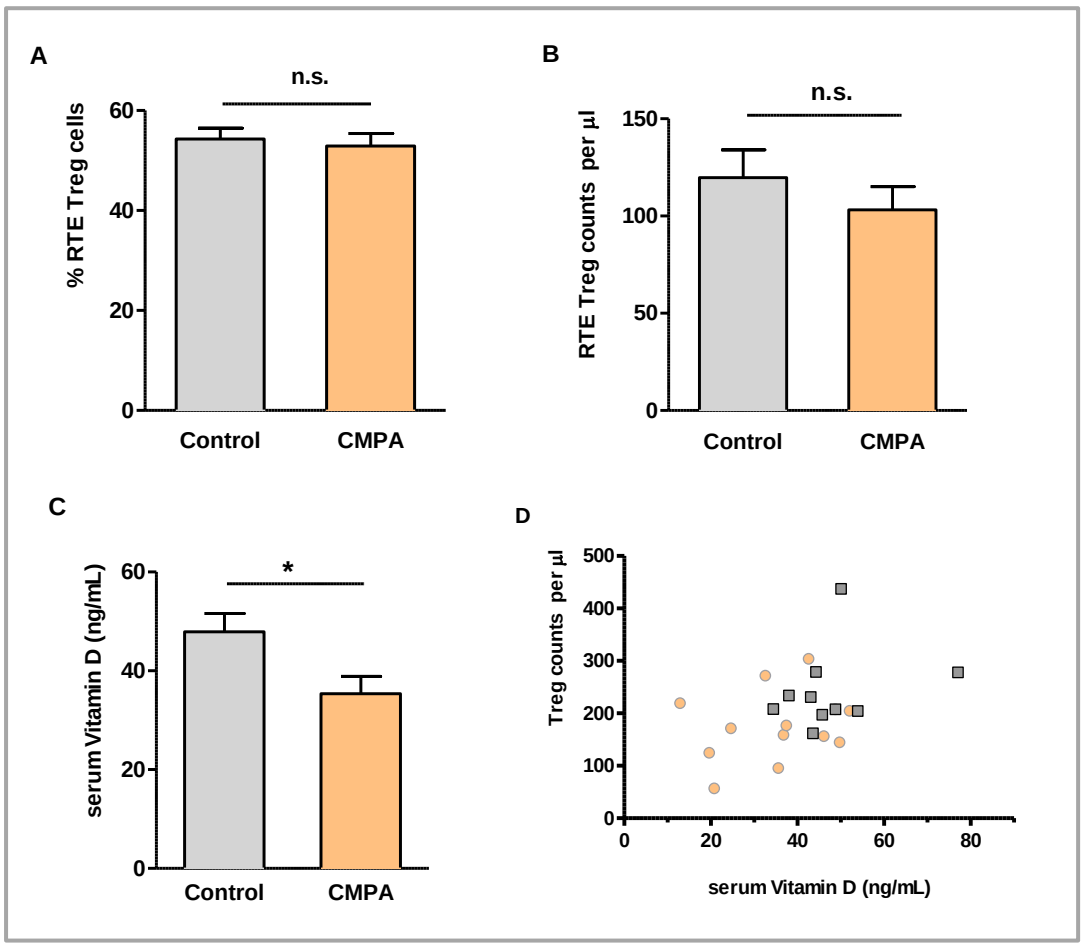

Figure 9. Percentage (**A**) and absolute counts (cells per µL of total blood) (**B**) of recent thymic emigrants (RTE) Treg cells. (**C**) Serum concentration of 25(OH)D (ng/ml) measured by chemiluminiscence. Median with interquartile range in both Control and CMPA groups are represented. n.s.: non-significant differences *: *P*<0.05 in non-parametric Mann-Whitney test comparing Control and CMPA values. (**D**) Correlation of Vitamin D levels and absolute counts of Treg cells (cells per µL of total blood). Control: grey squares; CMPA: orange dots. Pearson coefficient= 0.390; *P*=0.027.

Treg counts and serum Vitamin D levels can discriminate between Controls and CMPA children

Blood samples were obtained from patients with a suspicion of CMPA before the OFC and the definitive diagnosis of CMPA. Therefore, we analysed whether the three variables differentially expressed in CMPA children could be good predictors of the result in the oral challenge test and the clinical diagnosis of this allergy. The receiver operating characteristic (ROC) curve is widely utilized to evaluate the performance of diagnostic tests. The area

under the ROC curve (AUC) is a summary index commonly used to determine the quality to predict an event by different variables (Yao et al., 2015). ROC-curves were calculated to provide information on the sensitivity and specificity of these variables to discriminate between healthy Controls and CMPA children. The analysis of the data indicates that low serum 25(OH)D levels (AUC=0.754; 95% confidence interval (95CI)=0.553-0.955; *P*=0.041); low absolute number of Treg (AUC=0.728; 95CI=0.538-0.918; *P*=0.040) and a high frequency of IL4-TCD4 cells (AUC=0.747; 95CI= 0.547-0.946; *P*=0.037) are good variables to discriminate between CMPA and Control children (**Figures 10a-c**, respectively). The Treg/IL4-TCD4 counts ratio was also a good predictor (AUC=0.756; 95CI=0.536-0.946; *P*=0.027) to distinguish between CMPA and control children (**Figure 10d**).

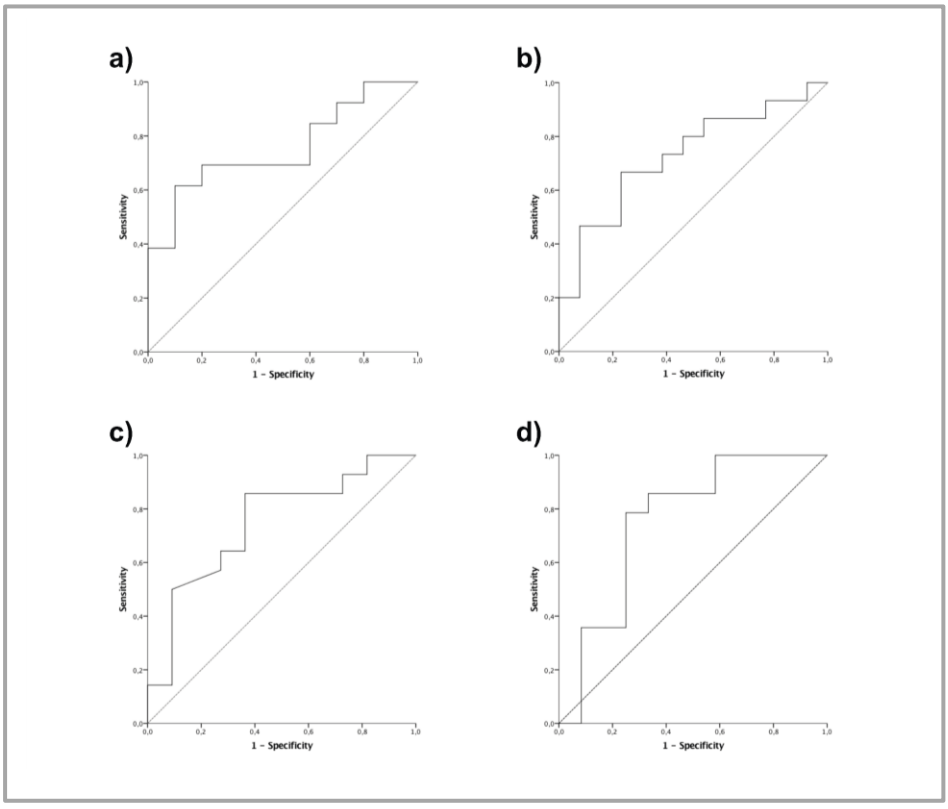

Figure 10. ROC Curves and predictive value of analysed variables: serum concentration of 25-hydroxyvitamin D (µg/L) (**a**); total Treg counts (cells per µL of total blood) (**b**); frequency of IL4-secreting CD4+ T cells (IL4-TCD4) (c); and the Treg/IL4-TCD4 counts ratio (**d**) were good discriminators between healthy and CMPA children.

Basal Vitamin D level was associated with the achievement of spontaneous tolerance to cow's milk in the first year.

Infants included in CMPA group were 1 year follow up to determine which patients spontaneously become tolerant to cow's milk. Seven out of 15 patients (46.6 %) remained allergic to cow's milk whereas 8 out of 15 (53.3 %) became tolerant or desensitized to cow's milk within the first year following the diagnosis. Interestingly, the vitamin D levels measured 1-4 days after the first adverse reaction to milk and before of CMPA diagnosis were good predictors of patients who spontaneously acquire tolerance or remain allergic. The presence of basal levels of vitamin D lower than 40 ng/mL predicted in our cohort those patients that remained allergic after 1 year, with a sensitivity of 87.5% and a specificity of 80% (AUC=0.850; 95CI: 0.608-1; *P*=0.040) (**Figure 11**).

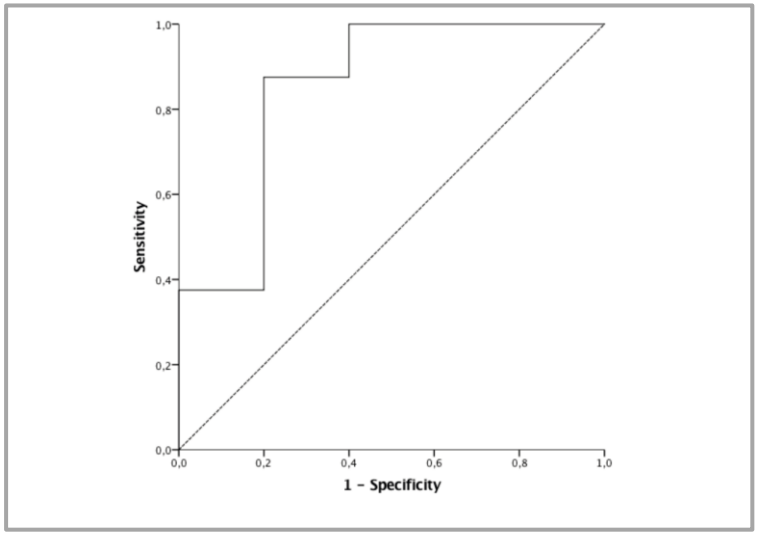

Figure 11. ROC Curve and predictive value of basal 25-hydroxyvitamin D levels (µg/L). Values of Vitamin D were a good predictor of those patients that did not acquire spontaneous tolerance to cow's milk along the first year after CMPA diagnosis.

4.1.2. DISCUSSION

In this study we performed an analysis of biomarkers which may elucidate immune alterations related with the establishment of cow's milk allergy in children. For that purpose, 15 infants with symptoms compatible with CMPA after cow's milk ingestion were recruited and the results compared with that of a group of 10 control patients from the same age-range. Samples were collected between 1-4 days after the first adverse reaction in CMPA children and therefore, we could study the immune system just after the onset of the allergic process.

Almost no difference was found in the frequency and absolute counts of the different immune cell subsets or in the phenotype of these populations between controls and CMPA children. It is noteworthy that the frequency of antigen-specific cells in peripheral blood is very low (allergen-specific T cells are typically less than 0.01%) (Wambre et al., 2014) and thus, the changes in allergen-specific cells were unlikely reflected in the values of cells we observed in peripheral blood. We did not analyze antigen-specific cells because the goal of our study was to identify immune markers easily measurable in peripheral blood that could be implemented in the clinical routine for the follow-up of these patients.

We observed a lower absolute number of naïve CD8+ T cells in CMPA children. Previous studies demonstrated a reduced percentage of CD8+ T cells in children with CMPA (Järvinen et al., 1998; Osterlund and Suomalainen, 2002), but they do not quantify the absolute counts of these cells. Because CD8+ T cells are one of the subsets responsible for the IFN-γ production, the reduced pool of naïve CD8 T cells in CMPA patients could be related with the decreased production of IFN-γ observed in patients with CMPA or other atopic diseases (Järvinen et al., 1998; Osterlund and Suomalainen, 2002). However, the role of CD8 T cells in food allergy is unclear with conflicting evidences of pathogenic or protective functions for this subset (Huber and Lohoff, 2015), and further studies must be conducted to clarify the function of CD8 T cells in CMPA allergy.

At the light of our results, a deficit in the number of Treg cells seems to be one of the determining factors related with the establishment of CMPA. Although the activation and/or differentiation of Treg cells appears to be correctly occurring in CMPA children, the frequency of IL4-TCD4 cells remains increased, probably as a consequence of the deficit in the number of circulating Treg cells that we observe in the CMPA group. In the context of food allergy, evidence from animal models and humans studies demonstrate how Treg cells can prevent allergic sensitization (van den Elsen et al., 2013) and induce oral tolerance (Karlsson et al., 2004; Fuentes Aparicio et al.,2013) and, thus, how Treg cells play a crucial role in the allergic disorders (Palomares et al., 2010). Our results indicate that the Treg deficiency is already present in the first 1 to 4 days after the first adverse reaction. Therefore, decreased Treg values could constitute a factor that predispose for the acquisition of an atopic phenotype and more concretely for the establishment of CMPA in infants. To definitively confirm this fact, it would be necessary to measure Treg values before the first ingestion of cow's milk, with difficulty in the large cohort of healthy infants should be enrolled for this kind of study as it will be unknown which children become allergic.

Regarding to the potential reasons for the Treg deficit in these infants, it is interesting to note that decreased numbers of Tregs are not due to the immaturity of the immune system at this age. Previous studies have demonstrated that neonates already have high Treg values (Correa-Rocha et al., 2012). In fact, controls infants of the present study showed Treg counts around 200 cells per µL, which are markedly higher than values observed in healthy children around 3 years old (Ferrando-Martínez et al., 2014) or 9 years old (Fuentes-Aparicio et al., 2013). We also found that, impairment in the thymic production of Treg cells can be discarded as responsible for the decreased Treg values. RTE Tregs were not different, and naïve Treg values were also comparable in both groups. That means that the deficit of Tregs are consequence neither of the level of thymic production nor of the arrival of these naïve Tregs to the periphery. Between the different Treg phenotypes, only the number of central memory and TemRA Treg cells was significantly reduced. These results indicate that the production, activation and differentiation to effector

cells in the Treg subsets seem to develop correctly in CMPA children. Indeed, we observed in the CMPA group that children with the highest values of circulating Treg cells were also those with lower frequencies of IL4-secreting CD4+ T cells, which reflects a suitable suppressive function of Tregs in these children. The fact that central memory and TemRA subsets of Treg cells (which are the most advanced steps of differentiation) are the most affected, could reflect a inaccuracy in the survival of these cells or in the mechanisms to maintain this pool after the antigenic stimulus.

Several studies demonstrate that vitamin D contributes significantly to the induction, survival and preservation of the Treg population (Penna et al., 2005; Chambers and Hawrylowicz, 2011; Ferrando-Martínez et al., 2014). Furthermore, numerous studies find a relationship between decreased values of vitamin D, in both mother (Chiu et al., 2015; Vijayendra Chary et al., 2015) and infant (Jones et al., 2015), and a higher incidence of allergy. The lower vitamin D values found in CMPA children and the direct correlation observed between vitamin D and the quantity of circulating Treg cells support the hypothesis that the impaired survival of Treg cells could be influenced by the deficit of vitamin D.

Our results show that IL4-secreting CD4+ T cells are the only population increased in periphery during the first phases of CMPA. Treg cells have been proved to specifically prevent an excessive expansion of CD4+ T cells at the mucosa that could lead to an allergic inflammatory response (Curotto de Lafaille et al., 2008). A great deal of evidence has also demonstrated that Tregs deficiency in the periphery is sufficient to evoke chronic T cell-mediated autoimmunity and immunopathology (Sakaguchi et al., 2008). Therefore, the deficit of Treg cells found in CMPA children could be enough to facilitate the persistence of IL4-producing cells, which could initiate and maintain the inflammatory cascade responsible for the allergic symptoms. A recent study also reports that Treg cell reprogramming toward a Th2-cell-like lineage can promote food allergy (Noval Rivas et al., 2015). The fact that the most differentiated subsets of Treg cells are notably decreased could also reflect a switch

of these cells to a Th2-like phenotype that will contribute to their increased frequency in CMPA children.

In summary, we hypothesized that after the introduction of cow's milk proteins in the diet, in those children with adequate immune homeostasis, Treg cells can prevent the inadequate expansion of IL4-producing CD4+ T cells. However, in children with a deficit of vitamin D probably Treg cells exerts its function initially, but without the appropriate stimulus (such as vitamin D) Tregs could have a reduced survival and become exhausted. In this scenario, the ratio of Treg/effector cells decreases and the inadequate suppression of effector cells will lead to the increased presence of IL4-secreting CD4+ T cells and the development of the allergic symptoms to these proteins. The demonstration that vitamin D sufficiency is an important protective factor for food allergy in the first year of life (Allen et al., 2013), supports the hypothesis that restoring the survival Tregs could be a potential strategy to prevent the establishment of CMPA in infants.

Finally, the statistical analysis indicates that low vitamin D values and decreased Treg numbers are good predictors to distinguish between controls and CMPA infants. The fact that these altered values are present few days after the first adverse reaction to the milk, prior to the definitive diagnosis of CMPA, support the utility of these values as diagnostic markers of CMPA. In addition, these parameters were good predictors of the results in oral challenge test. Thus, Treg values and serum vitamin D levels, which are easily measurable in a blood analysis, can be markers to discriminate between CMPA positive and negative and it could replace the use of oral challenge in those patients where these tests would involve a high risk. Finally, basal insufficiency of vitamin D was also a good predictor of those patients that will not achieve spontaneous tolerance in the first year, constituting also an interesting predictive marker of the clinical progression of these patients.

Further studies in larger cohorts of infants must be performed to confirm the quality of these markers, and whether Treg and vitamin D values in peripheral blood could constitute useful markers for the clinical follow up of CMPA patients.

4.2. Cow's milk and egg oral immunotherapy in children

Oral immunotherapy is nowadays one of the most promising approaches toward a treatment for food allergy. This work aims to investigate which are the immune mechanisms involved in the acquisition of oral desensitization by allergic children during a process of cow's milk or egg oral immunotherapy.

4.2.1. Oral long-course desensitization to cow's milk: Open-label, non-randomized, non-controlled study of cow's milk oral immunotherapy.

This study aimed to evaluate the safety and efficacy to induce clinical desensitization to cow's milk in a pediatric population following an open-label, non-randomized, non-controlled oral immunotherapy protocol based on an individualized up-dosing and characterized by a progressive introduction of milk-containing foods. In addition, the immune responses against β-casein of peripheral blood mononuclear cells from the allergic patients were evaluated before and after the protocol and compared to a non-allergic population.

The results reported in this section have been published in the reference: [Perezabad, L., Reche, M., Valbuena, T., López-Fandiño, R., Molina, E., López-Expóxito, I. (2017). Oral Food Desensitization in Children With IgE-Mediated Cow's Milk Allergy: Inmunological Changes Underlying Desensitization. *Allergy, Asthma & Immunological Research*. 9:35-42].

4.2.1.1. RESULTS

Patient characteristics and description of protocol

Twenty children (7 females and 13 males) aged between 1.5 and 11 years (mean 4.3 ± 0.54) and 15 non-allergic children (8 females and 7 males) aged between 5 and 14 years (mean 8.7 ± 1.05) were enrolled in the study (**Table 6**). Subjects were recruited from the Allergy Service at *Infanta Sofia Hospital* (Madrid, Spain).

All the CMPA patients enrolled were diagnosed through a compatible clinical history, positive SPT (≥ 3 mm of negative control) with CM, CN, α-LA and β-LG (performed with 5 mg/mL in all cases) and positive CM-, CN-, α-LA-, and/or β-LG-sIgE. The baseline average sIgE levels of the allergic group were 27.38 ± 6.97 kU/L (0.4-100 kU/L) for CM, 26.45 ± 7.45 kU/L (0.1-100 kU/L) for CN, 20.53 ± 5.98 kU/L (0.1-94.1 kU/L) for α-LA and/or 14.18 ± 5.0 kU/L (0.5-82.3 kU/L) for β-LG. In addition, all the subjects had experienced a positive reaction during a single blind food challenge (SBFC) with commercial semi-skimmed ultra-high temperature treated (UHT) pasteurized CM (3.3% protein) the month before the beginning of the study. Fifty five percent of the CMPA patients were allergic to other foods. Also, 45% of them had a past or current history of atopic dermatitis, 40% of asthma and 30% of allergic rhinitis (**Table 6**). Non-allergic children have no detectable IgE against a broad panel of the most common allergens. There were not statistically significant differences regarding sex or age between any of the groups.

Patient	Age (years)	Sex	FA	AD	RN	AS	SBFC Symptom	SBFC dose (mL CM)	Medication before OIT	OIT Symptoms	OIT duration (months)	Visits to the Unit (number)	Tolerance after OIT (mL CM)
1	1.5	F	No	No	No	No	Skin	120	No	Yes	24	10	≥ 200
2	5.9	F	No	No	Yes	Yes	Anaphylaxis	15	No	No	11	21	≥ 200
3	3.5	M	Yes	No	No	No	Anaphylaxis	4	Yes	Yes	20	37	≥ 200
4	2.4	M	No	No	No	No	Skin	64	No	Yes	14	27	≥ 200
5	3.8	M	Yes	Yes	No	No	Anaphylaxis	2	No	No	22	35	≥ 200
6	3	F	No	No	No	No	Skin	90	No	No	10	14	≥ 200
7	3.9	F	Yes	Yes	Yes	No	Anaphylaxis	0.3	Yes	No	18	42	≥ 200
8	3.1	M	No	No	No	No	Skin	70	No	No	6	16	≥ 200
9	7	M	Yes	No	Yes	Yes	Anaphylaxis	0.3	Yes	Yes	24	21	≥ 200
10	4	F	Yes	Yes	Yes	Yes	Skin	1	Yes	Yes	24	46	≥ 200
11	3.5	M	Yes	Yes	Yes	Yes	Anaphylaxis	4	Yes	Yes	24	43	≥ 200
12	3	F	No	No	No	No	Digestive	30	Yes	Yes	24	44	≥ 200
13	2.5	M	Yes	Yes	No	Yes	Anaphylaxis	4	No	No	20	24	≥ 200
14	4	M	Yes	No	No	Yes	Anaphylaxis	3	Yes	Yes	24	10	≥ 200
15	3.9	M	Yes	Yes	No	No	Anaphylaxis	0.1	Yes	Yes	24	37	60
16	3	M	Yes	Yes	No	No	Anaphylaxis	1	Yes	Yes	24	44	35
17	3.1	M	No	No	No	No	Respiratory	2	No	Yes	24	32	55
18	11	F	No	No	Yes	Yes	Anaphylaxis	4	Yes	Yes	24	48	80
19	10	M	Yes	Yes	No	Yes	Anaphylaxis	10	No	No	NA	NA	NA
20	4.1	M	No	Yes	No	No	Skin	30	Yes	Yes	NA	NA	NA

Table 6. Demographics, anamnesis, and response to CM-OIT in cow's milk-allergic patients.

Abbreviations: AD, atopic dermatitis; AS, asthma; CM; cow's milk; FA, other food allergies; NA, not applicable; OIT, oral immunotherapy; RN, allergic rhinitis; SBFC: single blind food challenge

The OIT protocol was carried out at the Allergy Day Unit under the direct supervision of the medical and nursing staff, and with all the equipment and material required for the treatment of possible allergic reactions that could occur during the procedure. CM-OIT protocol was performed with commercial semi-skimmed UHT CM, as described in **Table 7**. The starting dose of the OIT protocol was the previous one to the highest tolerated dose during the SBFC. Once the patients were able to tolerate 4 mL of undiluted CM without symptoms, an open challenge with baked goods containing milk was performed. Similarly, after tolerating 10 mL, patients were challenged with milk-containing cold meat, with milk chocolate after 15 mL, liquid fermented milk (Actimel®) after 60 mL, yogurt after 100 mL, cow´s cream cheese after 120 mL and, finally, with goat and ewe´s milk cheeses after 200-240 mL. Between visits, patients were advised to daily ingest at home the maximum dose tolerated during their last visit to the unit.

When needed, premedication with oral antihistamines was given to those patients that developed adverse reactions during the protocol, in order to control the symptoms. Reaction severity was assessed according to Clark and Ewan (2003). In the case of moderate reactions, these were pharmacologically treated and the protocol was restarted on the following week at the previously tolerated dose. Hence, the length of the protocol was increased stepwise depending on the severity of the reactions experienced by each patient. In the case of repeated severe reactions (anaphylaxis) the desensitization protocol was interrupted. Patients were considered to have successfully completed the OIT protocol if they were able to tolerate a minimum of 200 mL of CM in less than 24 months.

Once the patients completed the OIT protocol they maintained, during 1 year, a daily ingestion of 200 mL of commercial semi-skimmed UHT CM. If after 1 year the clinical desensitization was sustained, the patients were authorized to have a non-restricted diet.

Day	CM dilution	Dose (mL)	Challenge (% milk protein)
1	1/10	0.1	Commercial semi-skimmed UHT CM
8		0.2	
15		0.4	
22		0.8	
29	Undiluted	0.1	Commercial semi-skimmed UHT CM
36		0.2	
43		0.4	
50		0.8	
57		1	
64		2	
71		4	11 g baked goods (1.2% w/w)
78		10	15 g cold meat (2% w/w)
85		15	40 g milk chocolate (0.6% w/w)
92		20	
99		25	
106		30	
113		40	
120		50	
127		60	66 mL liquid fermented milk (3% w/w)
135		70	
142		80	
149		100	100 g yogurt (3.3% w/w)
156		120	75 g cow´s cream cheese (5.3% w/w)
163		150	
170		180	
177		210	
185		240	25 g goat and ewe´s cheese (30% w/w)

Table 7. Cow´s milk oral immunotherapy protocol (CM-OIT). Once the patients were able to tolerate 4 mL of undiluted CM without symptoms, an open challenge with baked goods containing milk was performed. Similarly, after tolerating 10 mL of CM, patients were challenged with milk-containing cold milk, with milk chocolate after 15 mL, liquid fermented milk after 60 mL, yogurt after 100 mL, cream cheese after 120 ml and, finally, with goat and ewe´s cheese after 240 mL.

Efficacy and safety of desensitization

The median threshold dose resulting in an allergic reaction during the SBFC was 4.0 mL (0.1-120 mL), with 60% of the patients developing anaphylaxis, 30% skin-related reactions, 5% digestive disorders and 5% respiratory complications (**Table 6**). Among the 20 patients included in the study, 14 (patients 1 to 14 in **Table 6**) tolerated more than 200

mL of CM, as well as goat´s and ewe´s milk cheeses, in an average period of 18.9 months [interval: 6-24 months], with 27.8 visits to the clinic of [interval: 10-46 times]. 57.14% of them experienced mild dermatologic reactions during the desensitization protocol, with the most common symptoms being mouth itching and perioral erythema.

Four patients (patients 15 to 18 in **Table 6**) tolerated between 35 and 80 mL of CM after 24 months of treatment and 40 visits (interval: 32-48 times) to the clinic, on average. At the end of the 24 month period, established as a time limit, this group was able to consume other foods containing milk as baked goods, cold meat and milk chocolate without developing adverse reactions. The reactions during desensitization of this particular group of patients were more severe, reporting strong abdominal pain and anaphylactic reactions. These children were considered partially desensitized. Two patients (patients 19 and 20 in **Table 6**) left the study for parental decision.

Thus far, patients have been keeping medical visits to assess their clinical status. After 36-48 months of follow up, with regard to patients completed the CM-OIT protocol, only one patient has shown severe adverse reactions, losing desensitization and being forced to eliminate dairy products completely from the diet. Between partially desensitized patients; one of them reached daily ingestion of 200 mL of CM and is following a dairy-free diet; other patient continues with partial tolerance (40 mL of CM and a half of yogurt); two of them stopped the desensitization protocol due to serious adverse reactions and are following a CM restricted diet. Taken together, nowadays, 70% of patients are following a free dairy diet and 5% have substantially increased the threshold dose, tolerating the equivalent dose of 40 mL of CM and a half of yogurt without developing negative symptoms.

Baseline status of cow´s milk allergic vs. non-allergic children

The immunologic status of the CM allergic children enrolled was initially assessed and compared with that of the group of non-allergic children of the same age range. As expected, CM allergic patients displayed levels of CM-, CM-, CN-, α-LA-, and/or β-LG-sIgE (baseline average sIgE levels are previously described) whereas almost non-existing sIgE levels were noticed in non-allergic subjects. Concerning the allergen-sIgG4 values, although non-statistically significant, a trend toward lower concentration of CN-sIgG4 was found in the allergic group (Mean ± SEM Allergic=2.15 ± 0.54; Mean ± SEM Control = 3.64 ± 1.29). CM-, αLA- and β-LG-sIgG4 data were not reported.

As presented in **Figure 12**, significant higher production of β-CN-specific Th2 cytokines (IL-13 and IL-5) was seen in the CM allergic patients compared with non-allergic children. In addition, a statistically significant increase was found for β-CN-specific IL-10 levels in the non-allergic group. Th1-related cytokines, IFN-γ and TNF-α were not detected.

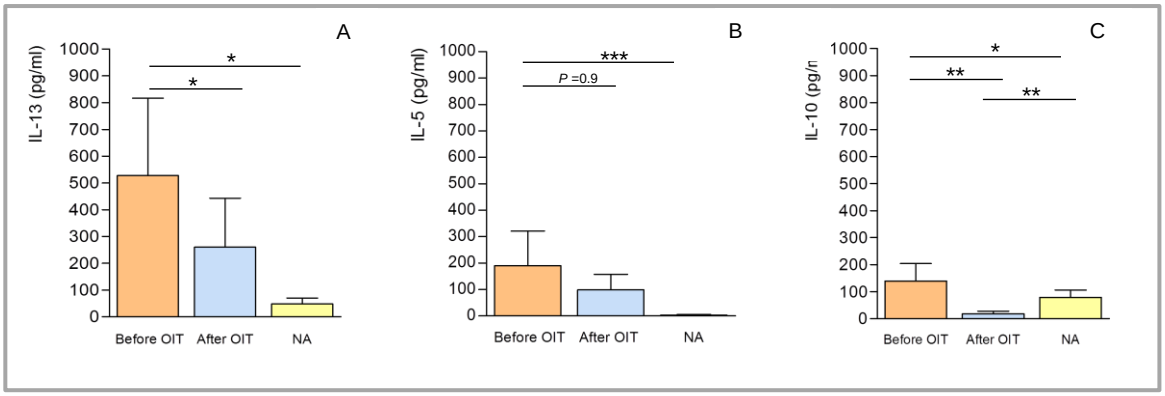

Figure 12. Cytokine production by (β-CN-stimulated PBMCs. Levels of (**A**) IL-13, (**B**) IL-5 and (**C**) IL-10 (pg/mL) before (orange blocks) and after (blue blocks) the OIT protocol in CM allergic patients that tolerated at least 200 mL of CM (n=14). Yellow blocks represent baseline cytokine production by β-CN-stimulated PBMCs in non-allergic children (NA; n=15). Bars represent mean ± SEM. ***: *P*<0.001, **: *P*<0.01,*: *P*<0.05.

Immunologic outcomes during OIT (CM-OIT outcomes)

***CM-OIT outcomes:* Specific antibody response**

As depicted in **Figure 13A**, in patients that successfully completed the CM-OIT protocol (N=14) a significant drop, of at least 4 fold, in their CM-, α-LA-, β-LG- and CN-sIgE levels was detected once the protocol was finished. Moreover, a significant increase from baseline was reached in the serum casein-sIgG4 concentration after the CM-OIT treatment (Mean = 1.77 ± 0.49 vs. 28.85 ± 12.42) (**Figure 13B**).

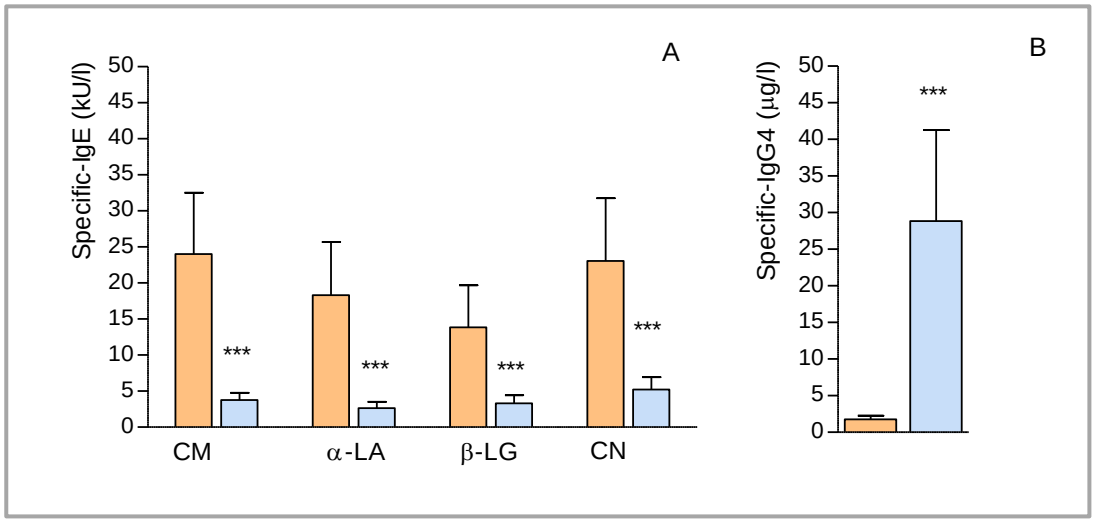

Figure 13. Antibody response in CM-allergic patients. Serum-specific (**A**) IgE (kU/L) and (**B**) IgG4 (µg/L) to CM, α-LA, β-LG and CN before (orange blocks) and after (blue blocks) the OIT protocol in patients that tolerated at least 200 mL of CM (N=14). Bars represent mean ± SEM. ***: *P*<0.001, **: *P*<0.01.

***CM-OIT outcomes:* β-CN-specific cytokine production by PBMCs**

Regarding the cellular response, no statistically significant difference was found in IL-5 (*P*=0.094) (**Figure 12B**), whereas a marked decrease in IL-13 (*P*=0.022) (**Figure 12A**) and IL-10 (*P*=0.002) (**Figure 12C**) were found between baseline and desensitization time points. Th1-related cytokines, IFN-γ and TNF-α were not detected.

Interestingly, the differences observed between allergic and non-allergic children in IL-13 (*P*=0.018) and IL-5 (*P*=0.000) before CM-OIT were no longer found once the protocol was completed (**Figure 12A** and **12B**). IL-10 production by β-CN-primed PBMCs from non-allergic donors was significantly higher (*P*=0.004) than that from CMPA patients at the end of the protocol (**Figure 10C**).

***CM-OIT outcomes*: Gene expression in β-CN-stimulated PBMCs**

We analyzed the expression levels of the transcription factors Foxp3, T-bet and GATA3 in the successful patients (N=14), before and after the CM-OIT protocol. The results did not show any significant changes, resulting in mean RQ values close to 1 of all studied genes. (Mean ± SEM and range for FoxP3 = 1.25 ± 0.13 [0.69-2.44]; T-bet = 1.92 ± 0.50 [0.33-6.28]), GATA3= 1.48 ± 0.30 [0.16-4.51]) (**Figure 14**).

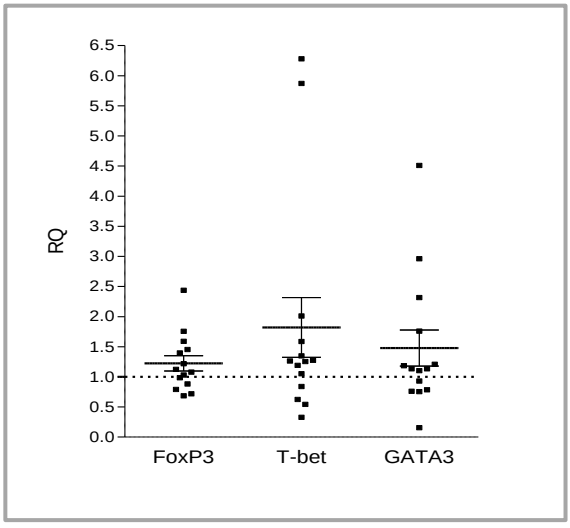

Figure 14. RQ values of transcription factors in response to intervention in complete number of CMPA patients completed CM-OIT (N=14). Black square represents change from baseline to successful OIT. RQ value of 1 means no difference in two times.

Baseline immunologic status of desensitized cow's milk allergic children vs partially desensitized

When comparing baseline CN-sIgG4 levels between patients successfully desensitized (N=14) and those that partially complete the protocol (N=4), higher levels were found in the second group (Mean ± SEM values: 1.77 ± 0.48 *vs* 10.12 ± 5.52). Moreover, it was noticed that 2 out of 4 of partially desensitized children displayed the highest baseline CN-sIgG4 levels of the total recruited participants (15.3 µg/L and 23.2 µg/L, patients 17 and 18 respectively in **Table 6**) and such values diverged strongly from the others. As a result, although without statistical significance, we suggest that high baseline sIgG4 level in CM allergic children could have an important clinical significance and maybe it could be considering as predictor of negative clinical response to OIT.

Regarding baseline levels of the different cytokines analyzed, there was a trend towards higher IL-5 (Mean ± SEM values: 190 ± 130.6 *vs* 29.93 ± 22.05) and IL-13 (Mean ± SEM values: 527.6 ± 289.4 *vs* 422.1 ± 209.4) after PBMCs stimulation with β-CN in the patients who successfully completed the protocol, albeit neither statistical nor clinical significance. IL-10 levels were only detected in one child of the partially desensitized group, nor Th1-related cytokines (IFN-γ and TNF-α) in either of the groups had detectable levels.

4.2.1.2. DISCUSSION

In the present study, CM desensitization (corresponding to a CM consumption ≥ 200 ml without trigger symptoms) was achieved by 70% of the children with IgE-mediated CM allergy enrolled in the study, in an average period of 18.9 months. At 36-48 months follow up, 70% of desensitized children were still consuming daily CM in the diet without restrictions. The desensitization rate was in the range of previous reports by *Meglio et al.* (2008), *González-Jiménez et al.* (2013), *Vázquez-Ortiz et al.* (2013), where 71.4%, 72% and 71.6% of the children enrolled were desensitized to CM, with 47% of the positive oral

food challenges being graded as anaphylactic reactions. Adverse reactions were usually controllable with a rate of occurrences lower than those reported in other CM-OIT protocols (Staden et al., 2007; Sánchez-García et al., 2012). In any case, the side effects encountered during the treatment considerably lengthened the duration of the protocol, compared to what was originally planned (185 days), highlighting the importance of adapting the dosing regimen to the patient's response to the therapy.

A distinct feature of the reported protocol is the progressive introduction of milk-containing foods into the patient´s diet: this allowed food diversification and helped to improve patient´s quality of life, while reducing the withdrawals from the therapy as the patients felt confident with the results of the intervention. It should be mentioned that desensitized children were also able to consume goat's and ewe's milk proteins. Several case reports of allergy to goat and sheep milk proteins in individuals previously desensitized to CM can be found in the literature (Rodríguez del Río et al., 2012; Tripodi et al., 2013). In fact, *Rodríguez del Río et al.* (2012) found, in patients who tolerated CM after CM-OIT, that 26% of them were still allergic to goat's and ewe's milk,

It is worth to mention that there are many methodological differences in the duration of the build-up phase between protocols. Rush schedules have demonstrated to be capable to rapidly desensitize patients to CM in a few days, confirming safety (Martorell et al., 2007; Longo et al., 2008; Staden et al., 2008; González-Jimenez et al., 2013), whereas, in an opposite way, some studies have documented that prolonged regimens enhance the desensitization effect, suggesting that longer treatment courses are more effective and possibly safer (Staden et al., 2007; Narisety et al., 2009; Meglio et al., 2013). Because of the length of the protocol and the strong familiar commitment required for CM-OIT, the study was open label and uncontrolled. However, the high baseline sIgE levels, as well as the adverse reactions observed during the therapy, suggested that spontaneous CMPA resolution was very unlikely in the population under study (Fiocchi et al., 2008; Wood et al., 2013). The absence of a placebo group also is a limitation of the study that could be justified by the results obtained in the placebo controlled trials performed by *Longo et al.*

(2008) and *Pajno et al.* (2010), in which none of the children included in the placebo group achieved even partial tolerance once the study had ended. It is likely that, in case that the treatment applied had altered the natural course of CM-oral tolerance achievement, it would have either anticipated it or increased the threshold dose for those patients that did not successfully complete the therapy (Staden et al., 2007; Meglio et al., 2008). While the current protocol setting cannot confirm patients were tolerant, as CM was not withdrawn for ethical reasons, it should be noted that all the patients considered successfully desensitized were on a free diet 36-48 months after completed the CM-OIT protocol.

Baseline sIgG4 levels increased along the therapy, as previously observed by a number of authors in other CM-OIT protocols (Skripak et al., 2008; Pajno et al., 2010; Bedoret et al., 2012; Keet et al., 2012; Savilahti et al., 2014a, Salmivesi et al., 2016) as well as in patients that spontaneously recover from CMPA (Savilahti et al., 2010; Lee et al., 2013), confirming the important role of this immunoglobulin in oral tolerance establishment. Concomitantly with the increase in sIgG4, a significantly reduced antigen-sIgE production was found. Although a decrease in allergen-sIgE production is commonly reported in most of the CM-OIT protocols described (Longo et al., 2008; Meglio et al., 2008; Zapatero et al., 2008; Martorell et al., 2011; Bedoret et al., 2012; Keet et al., 2012; García-Ara et al., 2013; Vazquez-Ortiz et al., 2013; Savilahti et al., 2014a) other studies have reported no change (Meglio et al., 2004; Skripak et al., 2008; Pajno et al., 2010) which, according to the authors, might be explained because of the shorter duration of their treatments. In fact, *Meglio et al.* (2008), followed up after 4 years and 8 months the CMPA children desensitized in their previous study (Meglio et al., 2004), reporting that the differences between CN- and αLa sIgE pre and post oral desensitization were no significant, whereas such differences between the pre desensitization and the long follow-up visit became significant for both allergen-sIgE.

In accordance with previous publications (Tiemessen et al., 2004; Tsuge et al., 2007), β-CN-primed PBMCs from CM allergic patients presented a significant Th2-biased phenotype when compared with not allergic individuals. In fact, enumeration of β-CN-

specific IL-4- and IL-13-secreting T cells has been proposed as a promising tool to improve diagnosis of CMPA (Michaud et al., 2014). β-CN (27% of the total milk proteins) was chosen for PBMC stimulation as it represents a serious health risk to patients with CMPA, since 75% of the sera from patients with IgE-mediated CMPA against whole bovine β-CN have IgE directed against it (Shek et al., 2005). Furthermore, it is known that PBMCs from clinically reactive IgE-mediated CMPA patients proliferate in response to LPS-free α_S, β and κ-CN, but not β-LG (Sletten et al., 2007). Importantly, baseline significant differences in IL-5 and IL-13 levels between CMPA and non-allergic children were no longer found once the treatment had finished, demonstrating a transition towards a non-allergic phenotype in the patients able to ingest ≥ 200 mL of milk without developing symptoms. To the best of our knowledge, there is only one other publication dealing with changes in the cytokine response by stimulated PBMCs from milk allergic individuals subjected to CM-OIT*. Bedoret et al.* (2012) found a shift from IL-4 and IL13 to IFN-γ production in the patients desensitized to milk. However, in our protocol detectable levels of β-CN-specific IFN-γ were not found. *Salmivesi et al.* (2016) found significant increases in serum IL-4 and IL-6 of CM allergic children after took part in a CM-OIT intervention. Same authors also reported no significant changes in the serum IL-5 and IL-10 concentrations. In a model of plasmacytoid dendritic cells (pDC)-CD4+ T cell co-culture, *Frischmeyer-Guerrerio et al.* (2014) showed reduced secretion of Th2 cytokines (IL-5 and IL-13) in response to CM from CM-OIT subjects, whereas secretion of IFN-γ and IL-10 to CM did not predict clinical responses in their study.

As well as other egg (Vickery et al., 2010) or peanut (Jones et al., 2009) OIT studies, IL-10 production by allergen-stimulated PBMCs decreased, suggesting, not only a Th2 cell impairment, but also a decreased Treg function; as IL-10 production is considered one of the main effectors responsible for the suppressive effect of Treg (Akdis et al., 1998; Akdis and Akdis, 2014). Interestingly, Bedoret et al. (2012) ruled out a role for allergen-specific FoxP3+ regulatory T cells in oral desensitization to CM, suggesting that, at least when high doses of antigen are administered, the mechanism subjacent lies on anergy or deletion, rather than suppression, of allergen-specific T cells. However, according to *Shreffler et al.*

(2009), allergen-specific and functionally suppressive Treg do play a role in the resolution of milk allergy and could be important targets for immune monitoring. These authors reported that introduction of milk into the diet causes a decline in the frequency of Treg present in the peripheral blood, in parallel with an increase in the IgG4/IgE ratio and a reduced basophile response; which they attributed to Treg cells being recruited to the gastrointestinal tract by allergen ingestion (Shreffler et al., 2009). Similarly, *Varshney et al.* (2011) claimed that decreased Th2 cytokine production and increased IgG4 and Treg cells are the main immunologic changes that accompany clinical efficacy of peanut OIT, even if they did not detect significant changes in blood IL-10; which raises the hypothesis that blood cytokine levels do not reflect mucosal production of Treg, or that mucosal and periphery Treg exert different functions. A further possibility is that induction of Treg is transient. Thus, *Jones et al*. (2009) found, in the course of peanut OIT, an early generation of Treg and an associated increased production of IL-10 by PBMCs that eventually decreased after 12 months. In this respect, it should be noted that, in our study, the long time period required for a successful outcome of the treatment (on average 18.9 months) might have masked certain immunological events. On the other hand and in accordance with our results, *Tiemessen et al.* (2004) reported that the CM-specific IL-10 production was significantly higher in T-cell clones derived from children with persistent CMPA compared with those from non-allergic children.

The lack of treatment-related changes in the expression of Treg (Foxp3), Th1 (Tbet), or Th2 (GATA3) transcription factors, despite the existence of measurable variations in cytokine production has been already reported in our studies of egg OIT, as in the study by *Jones et al.* (2009) with peanut OIT.

In conclusion, this report presents an efficient and safe CM-OIT protocol characterized by the progressive introduction of milk containing foods that may improves substantially the patient’s quality of life along the treatment course. Successful CM-OIT was accompanied by an immune alteration characterized by a significant increase in antigen-sIgG4 levels, as well as by a significant reduction in antigen-sIgE concentration and in IL-5 and IL-13

production by β-CN stimulated PBMCs, towards a non-allergic phenotype. More research needs to be done in order to understand the role of IL-10 in CM-OIT.

4.2.2. Oral rush desensitization to egg: A randomized controlled study of egg rush oral immunotherapy

The purpose of this randomized and controlled study was to evaluate the clinical and immune responses against egg and main egg allergens in a pediatric population with IgE-mediated egg allergy under a rush oral immunotherapy protocol based on a first 5-days rapid build up phase. In order to determine how these outcomes behave when the treatment is not used, we also evaluated such responses in a group of egg allergic children remained in egg exclusion diet. In addition, a group of non-allergic children were considered in this study, to set up and assess the immunological status of participants.

4.2.2.1. RESULTS

Subject population, inclusion and exclusion criteria

33 egg allergic children from both sexes, who followed an egg avoidance diet, including extensively heated egg, were invited to participate after informed consent from legal guardians. They were consecutively recruited between April and May 2012 at the Department of Allergy, *Hospital Infantil Universitario Niño Jesús*, Madrid, Spain, and the follow-up was completed in July 2013.

The inclusion criteria were:

1- Children between 5 and 18 years old.
2- Egg allergy diagnosis based on presence of IgE-mediated symptoms: positive SPT (mean wheal diameter ≥3 mm compared to negative control) and/or serum sIgE levels ≥0.7 kU/L for egg, egg white (EW), ovalbumin (OVA) and/or ovomucoid (OM).
3- Confirmed egg allergy diagnosis by positive DBPCFC to EW.

Participants were excluded if they had any of the following criteria:

- History of anaphylactic shock after egg consumption in the previous year.
- Severe or not controlled bronchial asthma.
- Non-IgE-mediated adverse events (AE) to egg.
- Eosinophilic esophagitis.
- Immunological diseases or malignant diseases.
- Any baseline disease contraindicating the use of epinephrine.
- Allergy to any component of the placebo.

Additionally, a group of 9 non-allergic children from both sexes and the same age range were included in the study.

SPT were performed and evaluated according to the standard procedures for prick testing (Dreborg and Frew, 1993) with egg (5 mg/ml), EW (1 mg/ml), OVA (1mg/ml) and

OM (1mg/ml) (Leti, Madrid, Spain). The allergen source used for DBPFC and egg ROIT was dehydrated EW (OVO-DES NM®, Nutrición Médica SL, Madrid, Spain) with entirely preserved allergenicity (Escudero et al., 2013). According to the manufacturer, 3600 mg of product is equivalent to one medium-sized EW and its protein content is 78% (2808 mg). DBPCFC doses were 4, 20, 50, 100, 225, 450, 900, and 1800 mg of dehydrated EW (cumulative dose of 2808 mg of protein).

Generation of study groups and description of protocol

As shown in **Figure 15**, 40 candidates for the treatment with a first egg allergy diagnosis based on presence of IgE-mediated symptoms (positive SPT and/or allergen-sIgE levels) were assigned into two groups, active group and control group, using a computer-generated randomisation table. Once performing the DBPCFC, after diagnosis based on full set of criteria, 33 candidates joined their group: Active group rush oral immunotherapy 1 (ROIT1; N=19), included those patients receiving egg ROIT immediately after randomization, and control group (CG; N=14) formed by those who were non-treated and continued on an egg avoidance diet during the next 5 months. Patients in CG who failed a second DBPCFC at 5 months joined to intervention receiving the same egg ROIT protocol as ROIT1 group. Thus, both ROIT1 and CG who become active, comprise the total number of patients that completed desensitization (N=30), study group named as rush oral immunotherapy 2 (ROIT2).

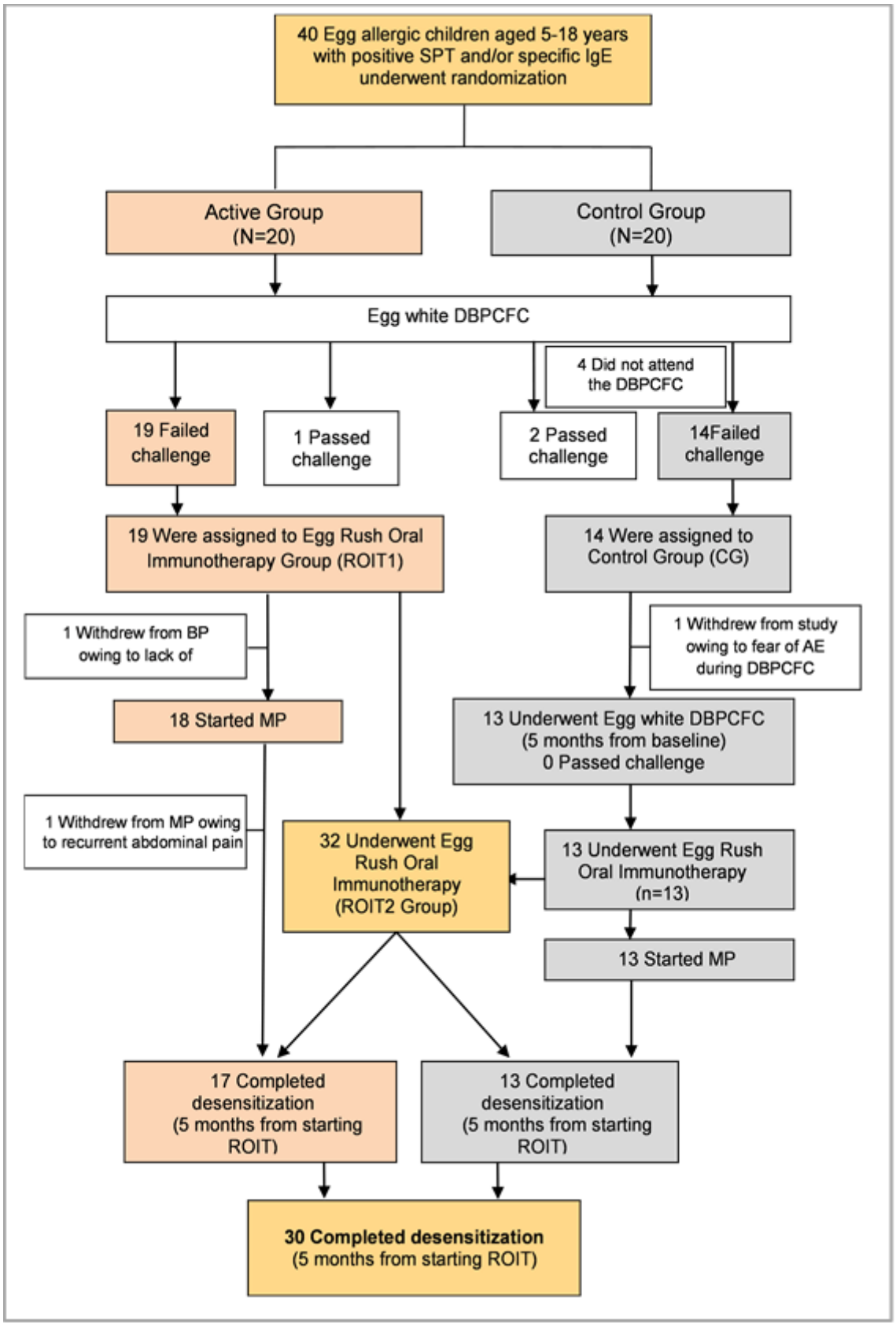

Figure 15. Study flow chart according to groups and interventions.

Abbreviations: BP, build up phase; MP, maintenance phase.

Blood samples were withdrawn at 3 different time points throughout the ROIT to perform the different analyses (**Figure 16**): T0: baseline; T1: 15 days from end of BP phase, and Tf: 5 months from baseline. Regarding the CG, those participants who failed the second DBPCFC at 5 months were enrolled in the ROIT intervention, being followed-up at same time points for 5 months. There were no significant differences between groups except for respiratory symptoms which were more frequently observed in the CG during DBPCFC (*P*=0.04) (**Table8**). Blood samples from non-allergic children were also analyzed to study the baseline allergic immunological status of all participants.

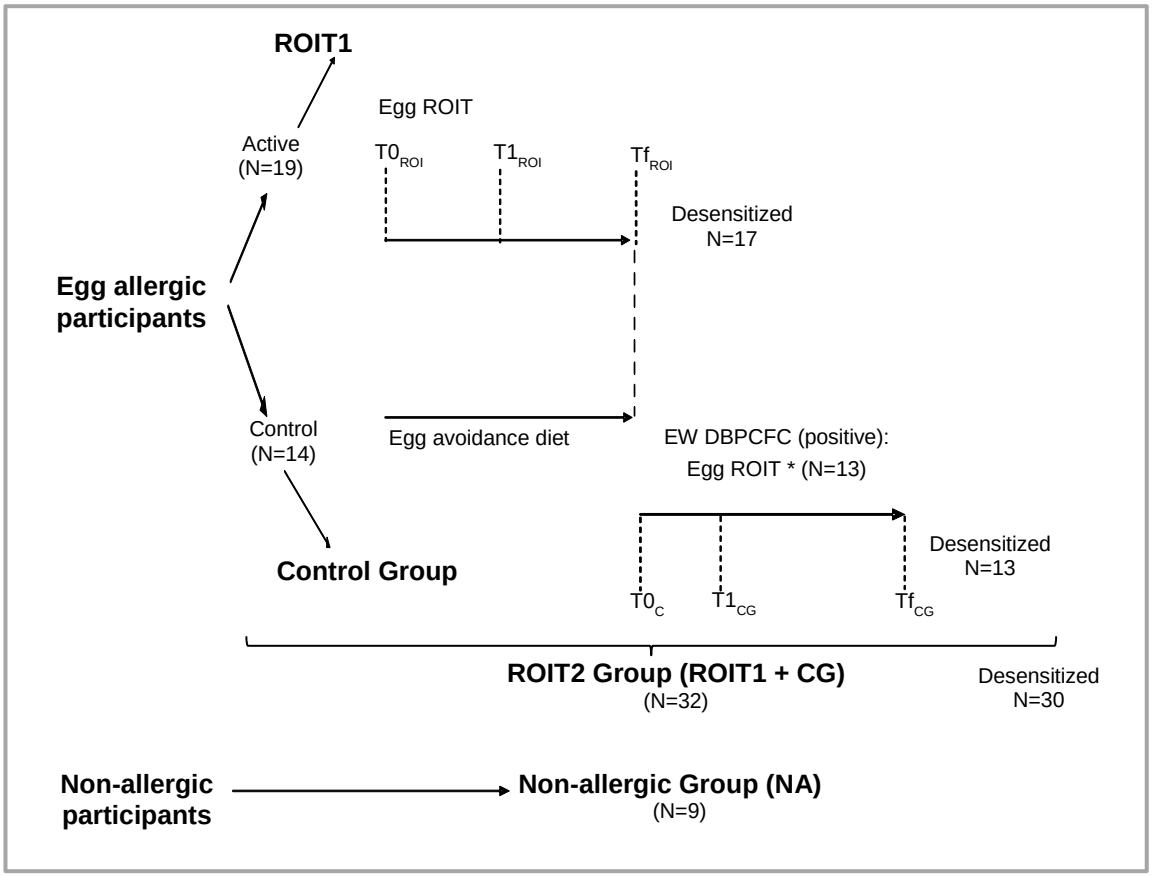

Figure 16. Schematic diagram depicting the time schedule of intervention and analyses performed. T0: baseline; T1: 15 days from end of build-up phase; Tf: 5 months from the baseline.

* Baseline of egg ROIT protocol in CG. Subjects who failed a second DBPCFC at 5 months joined the intervention and their data combined with ROIT1 to form ROIT2 group.

	TOTAL (N=33)	CG (N=14)	ROIT1 (N=19)
Male; N (%)	18 (54.6)	7 (50)	11 (57.9)
Age (years); mean (SD)	10,4 (2.6)	9.7 (2.3)	10.9 (2.7)
Age at first egg reaction (years); mean (SD)	12,9 (13.6)	15.5 (20.4)	11 (4.6)
Asthma (%)	57.6	27.3	30.3
Allergy to other foods (%)	69.7	85.7	57.9
Previous anaphylaxis (%)	42.4	37.1	31.6
Symptoms at baseline DBPCFC (%)			
• Cutaneous	20.9	17.8	23.6
Angioderma	4.2	6.7	2.0
Dermatititis	2.1	0	3.9
• Urticaria	14.6	11.1	17.7
• Oropharyngeal symptoms	30.21	31.11	29.4
• Digestive	29.17	26.67	31.4
• Conjunticvitis	1.04	0	2.0
• Respiratory	18.8	24.5	13.7
Rhinitis	14.6	15.6	13.7
Asthma	4.2	8.9	0
• Anaphylaxis	0	0	0
Threshold dose at DBPCFC (mg); median (range)	225 (20-3600)	225 (20-1800)	225 (50-3600)

Table 8. Baseline clinical characteristics and baseline symptoms at double-blind placebo-controlled food challenge according to study group.

The egg ROIT protocol designed consisted of 5 consecutive days build up phase (BP) (**Table 9**) consuming dehydrated EW on an outpatient basis, starting at the highest tolerated single dose in the baseline egg DBPCFC. The median threshold dose during the DBSBFC was 225 mg (range, 20-3600 mg) (**Table 8**). After 1 hour of observation without symptoms the subsequent dose was administered. In case of an adverse event, the previously tolerated dose was administered as the first dose on the following day. During the weekend, patients continued with the in-home daily of the last tolerated dose. Thus, BP doses were increasing gradually with a target of 3600 mg of dehydrated EW (2808 mg of EW protein; one medium-sized EW).

As a continuation of BP, subject continued the protocol until 5 months follow-up in a maintenance phase (MP), consisted of eating at home undercooked egg (undercooked fried egg, scrambled egg, or omelette) every 48 hours. In addition, children could freely take any other food products that contain egg proteins.

Positive DBPCFC Dehydrated EW (mg)	Day of ROIT	Number of doses	ROIT-starting dose of dehydrated EW [a] (mg)	EW protein (mg)
4	1	1	0.04	0.03
		2	0.08	0.06
		3	0.16	0.125
		4	0.32	0.25
		5	0.64	0.50
	2	6	0.4	0.31
		7	0.8	0.62
		8	1.6	1.25
20		9	4	3.12
		10	20	15.6
50	3	11	20	15.6
100		12	50	39
225		13	100	78
450		14	225	175.5
		15	450	351
900	4	16	450	351
1800		17	900	702
		18	1800	1404
	5	19	1800	1404
		20	3600 [b]	2808

Table 9. Egg ROIT protocol. 5-day build-up phase.

a :The allergen source used for DBPCFC and ROIT was dehydrated EW (OVODES NM, Nutrición Médica SL, Madrid, Spain). The ROIT starting dose was based on the eliciting dose threshold, so the build-up phase started with the highest single egg dose tolerated in the baseline DBPCFC. When the test result was positive with 4 mg, the starting dose was 0.04 mg. Doses were administrated in the hospital at intervals of 60 minutes. **b**: Equivalent to one medium-sized EW.

Efficacy and safety of desensitization

As shown in **Figure 15**, seventeen of 19 patients (89.5%) in the ROIT1 group completed BP and MP. Desensitization was defined as the patient´s ability to eat one

undercooked egg (fried, scrambled, or omelette) without or mild adverse events. One patient did not adhere to the protocol, failing eat more egg at the end of BP, and the other failed due to mild recurrent abdominal pain and refusal to eat egg after one month in MP (EW-sIgE levels of 25.4 and 112 kU/L, respectively). Thirteen of 14 (92.9%) patients in CG remained on an egg-avoidance diet for 5 months. One subject in this group dropped out before undergoing the second DBPCFC for fear of adverse events. None of the 13 patients in CG passed egg DBPCFC at 5 months (T3) (**Figure 16**).

Thus, after the enrolment of controls into the active treatment group (ROIT2; ROIT1+CG), we have data of 32 patients who underwent egg ROIT, 30 of whom were desensitized at 5 months of intervention (rate of success of 93.8%). 86.7% of the mentioned 30 patients completed the BP in 5 days or less (median 3 days; range 1-14 days). Concerning the safety of therapy, the median number of reactions per child in the BP was 2.5 (range, 0-17), being 54.8% of recorded symptoms gastrointestinal, 19.4% oropharyngeal symptoms, 11% cutaneous, 7.7% rhinitis, 5.8% bronchospasm, and 1.3% (2 episodes) anaphylaxis. In the MP most reactions were mild and local (75% oropharyngeal symptoms, 21.5% mild abdominal pain).

In order to evaluate the long-term efficacy, patients were contacted approximately two years after the end of study. We got information in 27 of children were desensitized (27/30) and, only 4 out of 27 (14.81%), reported any adverse event being not able to continue eating egg. Therefore, taking together all available data 24 months later from OIT, desensitization was maintained in 76.6% of patients (23/30).

Basal immunological status: egg allergic vs. non-allergic children

Previously to study the immunological effects of ROIT treatment in egg allergic children, a comparative study was perform in order to evaluate the basal immunologic status of those patients who completed desensitization (N=30) in comparison with the

group of non-allergic (NA) children (N=9). There were not significant differences regarding age and sex between the two groups at the inclusion in the study.

Table 10 shows the cytokine levels found in the supernatants of OVA-stimulated PBMCs in both studied groups. Results revealed that egg allergic children displayed a diminished Th1 cytokine profile in comparison with non-allergic children. PBMCs from allergic patients secreted significantly lower levels of both IFN-γ and TNF-α (*P*<0.001) than NA children. Regarding Th2-related cytokines, a diminished production of IL-5 and IL-13 was found in NA patients, although the differences did not reach statistical significance.

The Percentage of peripheral CD4+CD25+FoxP3+ T cells (Treg) were also measured in 16 egg allergic children and compared with those of children in non-allergic group (N=9). Non-statistically significant differences were found between groups (mean ± SEM: 7.49 ± 0.99 *Vs.* 5.20 ± 0.64) (*P*=0.160).

Cytokine	Egg allergic (N=30)	Non-allergic (N=9)	*P*-value
IL-10	320.32 ± 40.91	232.10 ± 43.78	0.224
IL-5	5.77 ± 3.18	0.07± 0.07	0.065
IL-13	25.67 ± 6.53	9.56 ± 2.35	0.176
IFN-γ	14.70 ± 5.64	594.72 ± 253.40	**<0.001**
TNF-α	64.78 ± 21.84	448.02 ± 110.70	**<0.001**

Table 10. Cytokine levels (pg/ml) after stimulation with OVA of PBMCs from egg allergic patients at the inclusion in the study compared with a group of non-allergic participants. Values indicate mean ± SEM. *P*<0.05 by Mann-Whitney 2-tailed test was considered significant.

Outcomes between groups: Active Group vs Control Group (ROIT vs. GC)

***ROIT vs. GC*: Skin prick testing and specific antibody response**

Values in skin prick tests (SPT) for egg, EW, OVA and OM, as well as from EW- OVA- and OM-sIgG4, were significantly lower (*P*<0.05) in ROIT1 than in CG. However, no

significant differences were found between the 2 groups in sIgE levels to egg, EW, OVA or OVM or total IgE. In ROIT1 but not in CG, SPT decreased significantly (**Figure 17A**) and $sIgG_4$ increased (**Figure 17B**) compared to baseline.

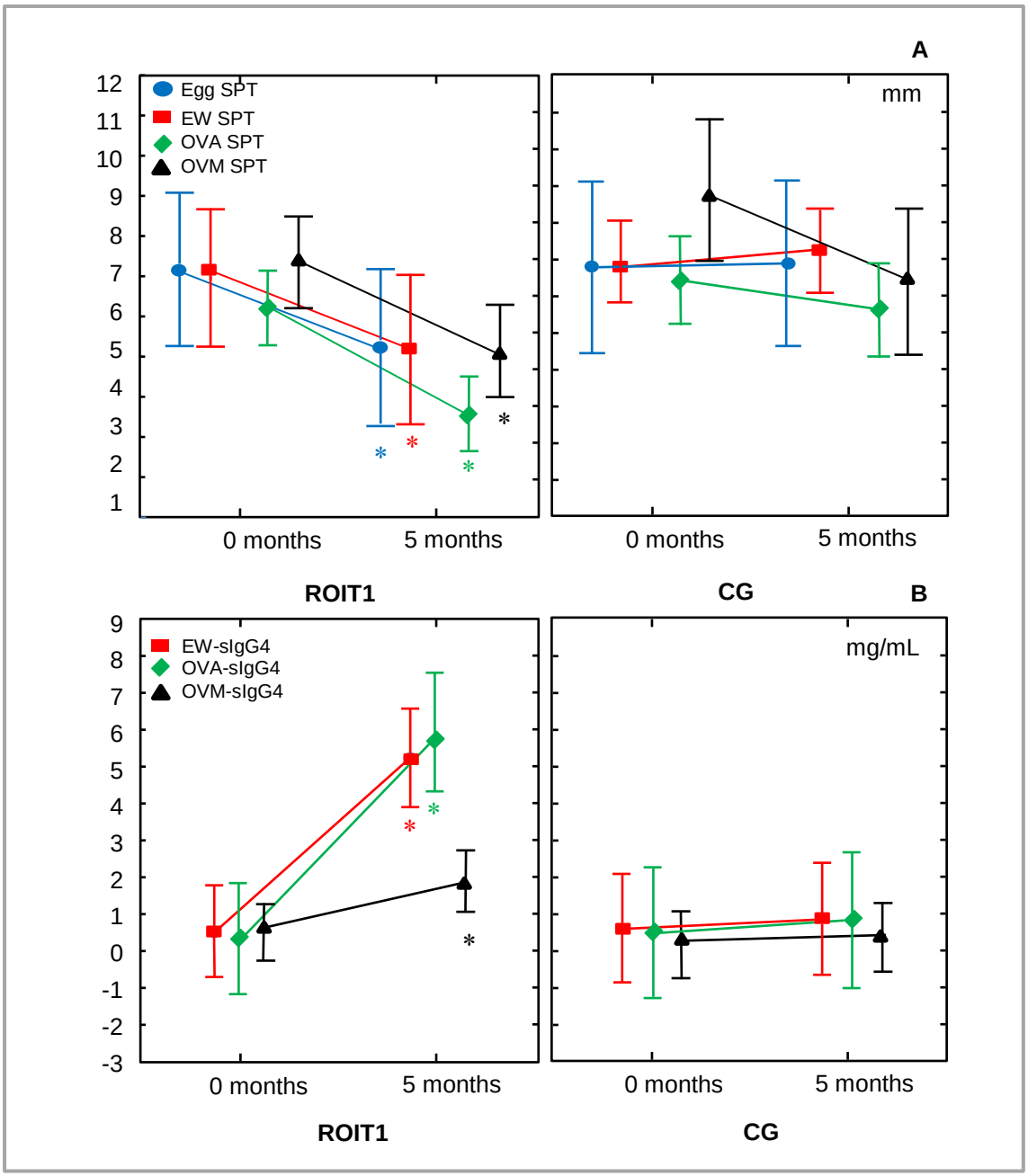

Figure 17. Mean levels of immune markers according to groups: CG and activeROIT1. (**A**) Skin prick tests (SPT) to egg protein fractions (*: *P*=0.05-0.001) and (**B**) Serum $sIgG_4$ (sIgG4) to egg protein fractions (*: *P*=0.05-0.001). Mean values, and two-sided test 95% confidence intervals.

***ROIT vs. GC*: OVA-specific cytokine production by PBMCs**

Comparison of cytokine levels in PBMCs cultured supernatants at the beginning of the study yielded not significant differences for any of the cytokines evaluated; demonstrating the immunological status of both groups of patients at the starting time was similar. On the contrary, as shown in **Table 11**, after 5 months of ROIT protocol, OVA-specific IL-13 concentration significantly decreased from baseline level (*P*=0.048) in the active group, whereas no changes in the CG patients were observed for OVA-specific IL-13. Levels of OVA-specific IL-5, IL-10, IFN-γ and TNF-α did not significantly change in either group.

Similarly, the percentage of peripheral CD4+CD25+FoxP3+ Treg cells did not showed statistically significant differences between baseline and 5 months course, in either ROIT1 (N=4; mean ± SEM: 6.59 ± 1.93 *vs.* 3.93 ± 0.94) (*P*=0.343) or CG subjects (N=6; mean ± SEM: 7.86 ± 2.32 *vs.* 7.52 ± 0.67) (*P*=0.844).

	CG (Egg avoidance diet) (N=13)			ROIT1 (Egg ROIT) (N=17)		
Cytokine	Baseline (0 months)	At 5 months	*P*-value	Baseline (0 months)	At 5 months	*P*-value
IL-10	320.5 ± 55.7	349.8 ± 79.6	0.999	297.7 ± 40.8	335 ± 107.7	0.623
IL-5	3.1 ± 2.0	6.8 ± 4.2	0.553	7.8 ± 5.4	3.5 ± 3.4	0.313
IL-13	17.6 ± 5.9	35.1 ± 14.7	0.086	31.9 ± 10.5	17.9 ± 12.4	**0.048**
IFN-γ	19.9 ± 8.1	76.5 ± 36.2	0.625	16.1 ± 8.0	9.9 ± 8.9	0.250
TNF-α	31.4 ± 14.4	351 ± 309.3	0.492	90.3 ± 36.2	126.1 ± 56.6	0.761

Table 11. Changes in the OVA-specific cytokines levels (pg/ml) from PBMCs of egg allergic patients included in the study, from baseline (0 months) to 5 months from randomization. CG: allergic patients who followed an egg-avoidance diet; ROIT1: allergic patients who received egg ROIT treatment. Values indicate mean ± SEM. *P*<0.05 by Wilcoxon 2-tailed test was considered significant.

***ROIT vs. GC*: Gene expression of transcription factors in OVA-stimulated PBMCs**

The expression of the transcription factors FoxP3, T-bet and GATA3 of OVA-stimulated PBMCs between the baseline and 5 months of study did not showed significant changes for neither of the groups, with mean of RQ values very close to 1 (Mean ± SEM [range] for ROIT1: FoxP3=0.76 ± 0.11 [0.27-1.96]; T-bet=1.14 ± 0.15 [0.43-2.68]; GATA3=1.17 ± 0.14 [0.29-2.60]) (Mean ± SEM [range] for CG: FoxP3=0.80 ± 0.08 [0.42-1.60]; T-bet=0.77 ± 0.15 [0.31-2.17]; GATA3=0.97 ± 0.14 [0.23-2.09]) (**Figure 18**).

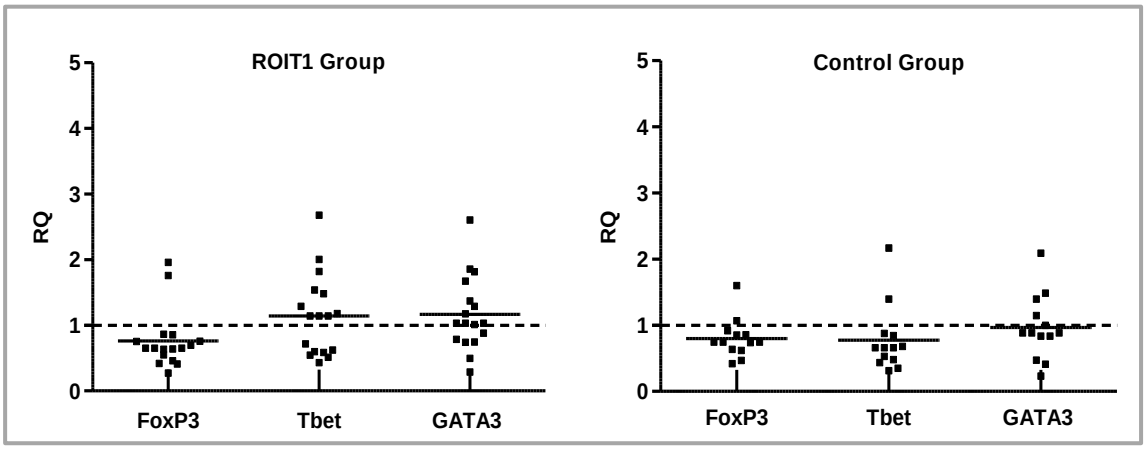

Figure 18. RQ values of transcription factors in response to intervention. Active group of egg allergic children (ROIT1; N=17) and control group following an egg avoidance diet (CG; N=13). Black square represents change from T0 (baseline) to Tf (5 months from baseline). RQ value of 1 means no difference between two times.

Immunologic outcomes during ROIT (Egg ROIT outcomes)

***Egg ROIT outcomes*: Skin prick testing and specific antibody response**

A significant decrease (P<0.001) in the prick test wheel size was observed for egg, EW, OVA and OM for all patients after the ROIT. In addition, a significant increase (P<0.001) in the serum-sIgG4 levels to egg protein fractions (EW, OVA, OM) was observed, together with a reduction in the serum-sIgE levels to mentioned egg protein

fractions (*P*=0.05-0.001). Specific IgE/IgG4 ratios also decreased significantly in all egg protein fractions (*P*<0.001). The decrease in SPT and sIgE/IgG4 ratio occurred earlier and was more significant than the changes in sIgE levels. These data are represented in **Figure 19**.

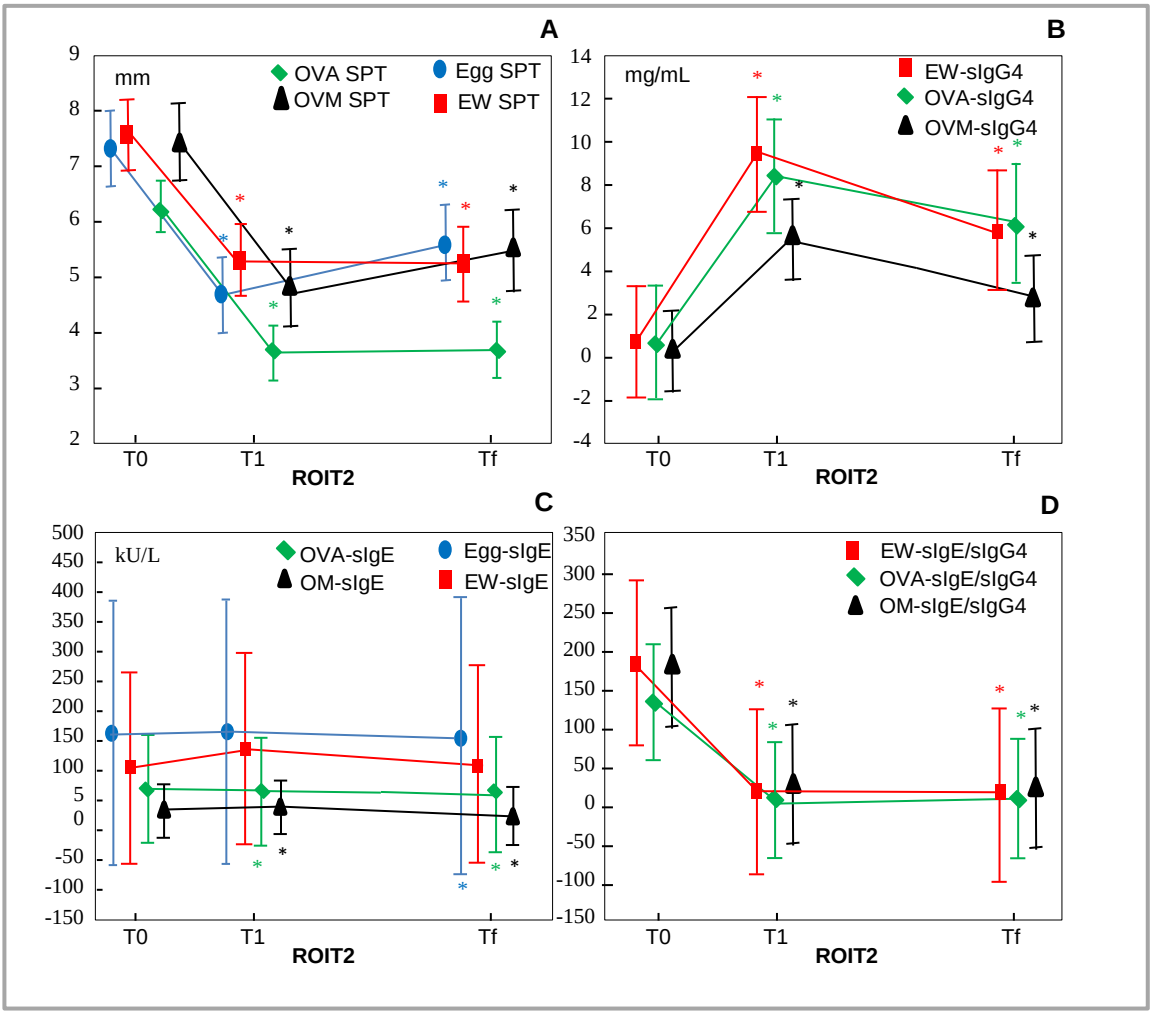

Figure 19. Mean levels of SPT, serum sIgE and serum sIgG4 in all children enrolled in the ROIT protocol (ROIT2; N=30) according to different times throughout the treatment. T0: baseline; T1: 15 days from end of build-up phase; Tf: 5 months from the baseline. (**A**) SPT to egg protein fractions (*: *P*<0.001); (**B**) Serum $sIgG_4$ to egg protein fractions (*: *P*<0.001); (**C**) Serum sIgE to protein fractions (*P*=0.05-0.001); (**D**) Serum sIgE/$sIgG_4$ ratio to egg protein fractions (*: *P*<0.001). Mean values and two-sided test 95% confidence intervals.

***Egg ROIT outcomes*: OVA-specific cytokine production by PBMCs**

The production of cytokines by OVA-stimulated-PBMCs was measured at baseline (T0), 15 days of the end of build-up phase (Tf) and 5 months from baseline (T3) in patients along the OIT protocol. **Table 12** shows the evolution of a panel of 5 allergen-specific cytokines during the ROIT treatment. Results revealed a diminished Th2 response when desensitization was completed, with a significantly lower concentration of OVA-specific IL-13 (*P*=0.098). Regarding Th1 cytokines, no significant changes were observed for OVA-specific IFN-γ, however, a tendency to lower TNF-α levels was found (*P*=0.08).

ROIT2 (N=30)						
Cytokine	T0	T1	*P*	T0	Tf	*P*
IL-10	320.3 ± 40.9	403.3 ± 48.3	0.104	320.3 ± 40.9	299.7 ± 62.7	0.435
IL-5	7.4 ± 3.5	2.9 ± 1.4	0.376	7.4 ± 3.5	2.7 ± 2.0	0.160
IL-13	33.2 ± 8.6	34.6 ± 7.2	0.875	33.2 ± 8.6	14.8 ± 7.3	**0.098**
IFN-γ	42.2 ± 16.9	66.9 ± 33.0	0.502	42.2 ± 16.9	18.3 ± 10.7	0.99
TNF-α	203.2 ± 134.7	122.3 ± 32.6	0.856	203.2 ± 134.7	114.6 ± 46.3	0.080

Table 12. Cytokine levels (pg/ml) after stimulation with OVA of PBMCs from all the allergic patients completed desensitization (ROIT2). T0: baseline; T1: 15 days from end of the build-up phase; Tf: 5 months from baseline. Values indicate mean ± SEM. *P*<0.05 by Wilcoxon 2-tailed test was considered significant.

***Egg ROIT outcomes*: OVA-specific Treg production by PBMCs**

Percentage of peripheral CD4+CD25+FoxP3+ Treg cells was measured in 10 patients who completed the desensitization protocol, but statistically significant differences did not occur between baseline (T0) and the end of treatment (Tf) (mean ± SEM: 6.59 ± 0.89 *Vs.* 5.39 ± 0.66) (*P*=0.160) (**Figure 20**).

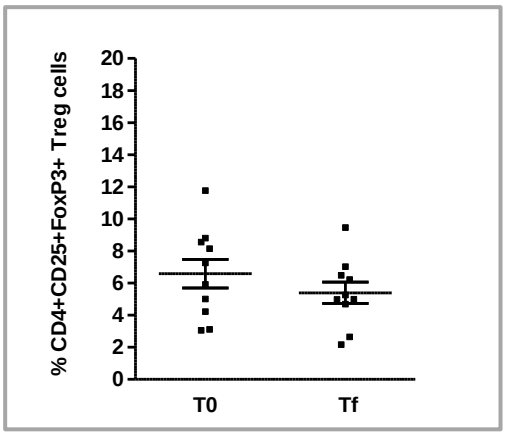

Figure 20. Percentage of CD4+CD25+FoxP3+ Tregs cells after stimulation with OVA of PBMCs from 10 allergic-patients who completed desensitization: T0: baseline; Tf: 5 months from baseline. Values indicate mean ± SEM. *P*<0.05 by Wilcoxon 2-tailed test was considered significant.

***Egg ROIT outcomes*: Gene expression in OVA-stimulated PBMCs**

RT-qPCR analysis of the transcription factors FoxP3, T-bet and GATA3 for all the comparisons mentioned revealed no significant changes with a mean of RQ values very close to 1 in all cases (Mean ± SEM [range] of RQ between T0 and T1: FoxP3=1.12 ± 0.13 [0.22-4.30]; T-bet=1.35 ± 0.04 [0.49-4.34]; GATA3=1.27 ± 0.99 [0.46-3.77]) (Mean ± SEM [range] of RQ between T0 and T3: FoxP3=0.96 ± 0.13 [0.27-3.93]; T-bet=1.25 ± 0.13 [0.29-3.22]; GATA3=1.13 ± 0.11 [0.38-2.68]) (**Figure 21**).

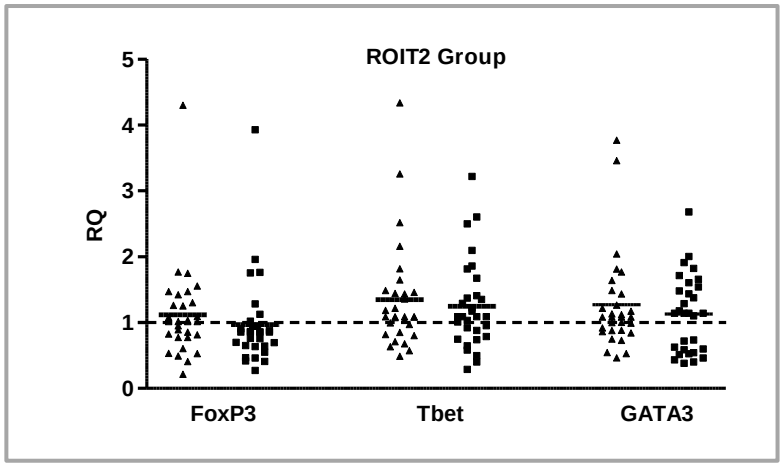

Figure 21. RQ values of transcription factors in response to intervention in complete number of egg-allergy patients completed ROIT (ROIT2; N=30). Black square represents change from T0 (baseline) to T3 (5 months from baseline); black triangle represents change from T0 to T1 (15 days from end of build-up phase). RQ value of 1 means no difference in two times.

4.2.2.2. DISCUSSION

The ROIT intervention employed in the present study was clinically effective, with a 94% of patients desensitized and being able to ingest 1 egg after 5 months of therapy. This outcome is similar to the percentage achieved with other published randomized controlled egg OIT studies, which also used dehydrated EW as allergenic source (Caminiti et al., 2015; Escudero et al., 2015). However, this study used a rush desensitization protocol in which the BP phase (tolerance of equivalent of 1 EW) was completed in as few as 5 days, whereas *Escudero et al*. (2015) reported a prolonged phase of 30 days. In *Caminiti et al*. (2015) used an equivalent dose of 1 EW, and desensitization was achieved even through a longer period (120 days).

To our knowledge, most published egg OIT studies included a BP of around 60 days (Ibáñez et al., 2015) and only a few rush oral desensitization to egg protocols have been published (Itoh et al., 2010; García Rodríguez et al., 2011), neither of which included randomized design nor a control group. These studies used cooked egg during the MP and

results showed a loss of desensitization during this period. There is controversy about the impact of using either raw or cooked egg on OTI efficacy (Praticò et al., 2014). In the current study, egg was given undercooked (nearly raw) during the MP in order to preserve its allergenicity, and the model of intervention was found to be effective, considering that 96.8 % of children reached MP and were able to continue the maintenance dose at the scheduled 5 months. The shorter treatment duration improves the adherence to the protocol for both children and parents, and also make easier to differentiate actual effects of intervention from natural outgrowth.

As inclusion criteria, the egg allergic children enrolled in this study showed detectable serum sIgE for egg protein, but in order to better assess the baseline immune status of the allergic children we also recruited a population of non-allergic children of the same age range with which the allergic were compared. In this comparison, egg allergic subjects showed a decreased Th1 cytokine response pattern by OVA-stimulated PBMCs with significantly lower production of IFN-γ and TNF-α. Therefore, children selected to ROIT reported a T-cell response clearly differentiated from non-allergic status, and based on the cytokine profile we could predict the allergic status of the participating subjects.

In this study, the decrease of OVA-specific IgE was very significant in desensitized patients. Other egg OIT protocols displayed lower antigen-specific IgE levels at the end of the immunotherapy (Vickery et al., 2010; Dello Iacono et al., 2013; Meglio et al., 2013), also those studies with a rush protocol (Itoh et al., 2010; García Rodríguez et al., 2011). However, no significant changes in IgE levels during OIT have also been reported (Burks et al., 2012; Fuentes-Aparicio et al., 2012). This fact, could be explained by the dynamic immunologic response of IgE through development OIT. The mechanism by which allergen-specific IgE changes are still under investigation. Some studies have shown that OIT alters the binding pattern of antigen to IgE, possibly through changes in the diversity of epitope recognition or altered antigen affinity (Reviewed in Wood, 2016). On the other hand, human IgG consists of four subclasses IgG1-4, which are functionally diverse and differentially regulated. In the present study a significant increase in the serum sIgG4 to

egg protein fractions (EW, OVA OM) was observed, however, we are conscious of our study did not measure other different serum OVA-specific subclasses apart from IgG4 which are discussed of being relevant in food allergy in recent years (Salmivesi et al., 2016; Sugimoto et al., 2016;). In their study of egg ROIT, *Sugimoto et al.* (2016) suggest that a significant rise in allergen-specific IgG1 levels after the rush phase of OIT could be a useful biomarker of positive response to egg OIT.

Although participants in the study might spontaneously outgrow their egg allergy we believe this to be unlikely. First, almost all of the egg allergic children formed the CG did not pass the oral food challenge at 5 months. Second, none of the children in the CG were able to eat a regular serving of egg after 5 months of study, compared to 89.5% of those receiving ROIT (ROIT1) and completed desensitization. Third, ROIT resulted in a reduction in the OVA-specific IL-13 levels in ROIT1 subject whereas no changes in the CG patients were observed for this cytokine, supporting that this changes in OVA-specific IL-13 status are as a results of the therapy, but not of spontaneous tolerance. We also proved that there were no significant differences between the active group (ROIT1) and the control group (CG) in the baseline cytokine profiles (data not shown).

When immunotherapy with an allergen is effective, it appears to be related with a shift from Th2 cytokine production (characteristic of allergic status) toward a Th1 (non-allergic) profile (Berin and Shreffler, 2016; Burbank et al., 2016; Wood, 2016). In these T-cell responses during the course of OIT is involved an induction of Treg cells (Fuentes-Aparicio et al., 2014; Syed et al., 2014; Berin and Shreffler, 2016). Although results of *Fuentes-Aparicio et al.* (2014) showed that egg desensitization was related to a significant increase in the frequency and absolute counts of Treg cells (measured as CD4+CD25+FoxP3+ T cells), in the present study we did not find statistically significant differences in the percentage of peripheral Treg cells between baseline and the end of treatment, neither did in the OVA-specific IL-10 production in which Treg cells are also involved. Previous studies have reported a significant increase of IL-10 levels after successful OIT (Jones et al., 2009; Vickery et al., 2010; Syed et al., 2014). However, although it was not significant, in this

ROIT protocol OVA-specific IL-10 levels decreased in children completed egg desensitization from baseline to 5 months of intervention. This outcome is consistent with findings by *Itoh et al.* (2010) in their protocol of rush egg OIT and it was also reported in *Fuentes-Aparicio et al.* (2012), in which serum IL-10 levels also suffered a reduction after OIT. Thus, the role of IL-10 when egg desensitization is induced needs yet to be clarified.

Together with IL-10 levels, the release of other key cytokines involved in the Th1/Th2 balance seems to be an important factor for oral tolerance. Upon allergen-specific stimulation, a reduction in the allergen-specific Th2-related cytokines levels (IL-4, IL-13, IL-5) and a rise in those of specific Th1-related cytokines (IFN-γ TNF-α) is the pattern of cytokine levels expected to detect after successful OIT (Berin and Shreffler, 2016). However, the conclusions we can extract about this concern from available OIT reports are controversial (Jones et al., 2009; Vickery et al., 2010; Varshney et al., 2011; Syed et al., 2014; Wisniewski et al., 2015; Salmivesi et al., 2016). In the current study, we were able to demonstrate a reduction in OVA-specific IL-13 after immunotherapy in patients completed desensitization. In addition, changes in OVA-specific IL-13 at 5 months of our egg OIT protocol separated the immune response of the egg allergic children in the active group (ROIT1) from those in the CG. Nevertheless, apart from a significant decrease of IL-13, we could not report any other significant differences for the cytokines tested. We could implicate the high inter-individual variation, the undetectable levels of some cytokines at baseline and the limitation for collecting data at more times during the curse of OIT. This later point is particularly important due to the potentially transient clinical efficacy of OIT as reported by *Gorelik et al.* (2015) in their peanut OIT.

This study neither was able to demonstrate changes in the expression of the transcription factors FoxP3 (Treg response), Tbet (Th1) and GATA3 (Th2) in response to intervention, nor in the expression of control group. There is a lack of studies reported gene expression of PBMCs from allergy subjects under allergen-specific stimulation.

The described approach has certain limitation when confirming tolerance to egg. The efficacy of a OIT protocol depends on the defined endpoint, which could be to induce

desensitization alone or a more durable state of clinical tolerance often referred to as "sustained unresponsiveness" (Burbank et al., 2016). To support this ultimate aim of the food allergy treatment, the ability to tolerate the food after discontinuing ingestion of the allergen for a period of at least 4-12 weeks must be confirmed (Rolinck-Werninghaus et al., 2005). *Burks et al.* (2012) reported in their randomized controlled study with long-term follow-up that, 75% of participants passed the oral food challenge at 22 months (10 g of dehydrated EW), but only 28% demonstrated "sustained unresponsiveness" on the re-challenge, after being on a subsequent avoidance of egg consumption for 6 to 8 weeks. Similar outcome is described in *Caminiti et al.* (2015), where egg desensitization occurred in almost all subject while only 1/3 of them passed the food challenge after an egg avoidance phase of 3 months. Therefore, to determine the extent to which the protective effect requires continued consumption becomes a prime focus of interest in OIT. In the present study, efficacy is defined as desensitization achievement, namely patient´s ability to eat one undercooked egg (fried, scrambled, or omelette) without or mild adverse events. This desensitization procedure is deemed as very successful with a rate of success of 93.8% (30/32) and the long-term efficacy of this state is also reported two years after the end of study in the 76.6% of desensitized patients (23/30), however, "sustained unresponsiveness" after a period of egg avoidance and later re-introduction of food is not confirmed as patients have not stop eating eggs once desensitized.

In conclusion, the present study shows that the proposed rush OIT protocol can induce desensitization to egg proteins in school age children in few days with high efficacy. The results also suggest that the significant decrease in OVA-specific IL-13 at short time of immunotherapy could be a useful biomarker of positive response to egg OIT. Although further studies involving a higher number of patients and analyzing a wide range of biomarkers are needed, we consider that this therapy could replace egg avoidance as the therapy for egg allergy. However, to confirm the "sustained unresponsiveness" and the development of post desensitization strategies that promote long-term immune tolerance become an essential prerequisite.

4.2.3. Oral long-course desensitization to egg: Open-label, non-randomized, non-controlled study of egg oral immunotherapy.

The purpose of this study was to establish differences in the basal immunologic responses between an egg allergic group of children and a population of non-allergic children. We also investigated the safety and efficacy of a specific egg OIT protocol for inducing clinical desensitization, analyzing the associated immune responses too. An open-label, non-randomized, non-controlled study was designed, characterized by an individualized up-dosing with introduction of egg-containing foods into children diet.

The results reported in this section have been published in the reference: [Perezabad, L., Reche, M., Valbuena, T., López-Fandiño, R., Molina, E., López-Expóxito, I. (2015). Clinical efficacy and immunological changes subjacent to egg oral immunotherapy. *Annals of Allergy, Asthma & Immunology*. 114:504-509].

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4.2.3.1. RESULTS

Patient characteristics and description of protocol

Twenty egg allergic patients (11 males and 9 females) aged between 5 and 15 years (mean ± SEM= 10.8 ± 0.71 years) and 15 non-allergic children (7 males and 8 females) aged between 5 and 14 years (mean ± SEM= 8.7 ± 1.05 years) were enrolled in the study. Subjects were recruited from the Allergy Service at *Infanta Sofia Hospital*, San Sebastian de los Reyes, Madrid.

All the children enrolled in the egg allergic group were patients diagnosed through a compatible clinical history, positive SPT (≥ 3 mm of negative control) with egg (5 mg/mL), EW (5 mg/mL), OVA (5 mg/mL), OM (5 mg/mL) and LZ (10 mg/mL) and positive sIgE for EW, yolk, OVA, OM and/or LZ. In addition, all the subjects had a positive reaction during a SBFC with pasteurized liquid EW (8.3% protein; Huevos Guillén, Valencia, Spain) during the month before the beginning of the study. The average dose of pasteurized EW that elicited allergic reactions during the SBFC in the egg allergic group was 1.56 mL [range: 0.001-8.0 mL], with 45% of the patients developing skin-related reactions, 30% anaphylaxis and 25% digestive symptoms. None of the recruited children experienced respiratory problems during the SBFC (**Table 13**). All but 3 egg-allergic subjects had allergies to environmental aeroallergens. Also, a 65% of them were allergic to other foods different than egg. Egg allergic patient's demographics and anamnesis are described in **Table 13**.

Non-allergic children included in the study showed no detectable IgE against a broad panel of the most common allergens. There were not statistically significant differences regarding age and sex between allergic and non-allergic groups.

Patient	Age (years)	Sex	AA	FA	SBFC Symptom	SBFC dose (mL EW)	Medication before OIT	OIT Symptoms	OIT duration (months)	Tolerance after OIT (mL EW)
1	12	F	Yes	Yes	D	4.0	Yes	Yes	12	32
2	5	F	No	Yes	S	0.01	Yes	Yes	14	32
3	6	M	No	No	S	4.0	No	No	6.0	32
4	5	M	Yes	Yes	S	2.0	No	Yes	12	32
5	15	M	Yes	Yes	A	8.0	No	No	6.0	32
6	12	M	Yes	Yes	S	0.1	Yes	Yes	18	32
7	14	M	Yes	Yes	S	0.5	No	No	6.0	32
8	10	M	Yes	Yes	D	0.01	Yes	Yes	12	32
9	14	F	Yes	Yes	S	1.0	Yes	Yes	12	32
10	11	M	Yes	No	S	0.05	No	No	8.0	32
11	11	F	Yes	No	A	0.2	Yes	Yes	20	32
12	13	M	No	Yes	A	0.06	No	No	15	32
13	10	F	Yes	No	A	0.1	Yes	Yes	24	2.0-10
14	9	M	Yes	Yes	D	0.06	Yes	Yes	18	2.0-10
15	9	M	Yes	Yes	A	0.001	Yes	Yes	18	2.0-10
16	14	F	Yes	Yes	S	0.01	Yes	Yes	24	2.0-10
17	15	F	Yes	Yes	S	0.1	No	Yes	18	<2
18	14	M	Yes	No	D	8.0	Yes	Yes	9.0	<2
19	8	F	Yes	No	A	1.0	Yes	Yes	7.0	<2
20	10	F	Yes	No	D	2.0	No	NA	NA	NA

Table 13. Demographics, anamnesis, and response to egg OIT in egg allergic patients. Abbreviations: A, anaphylaxis; AA, allergies to aeroallergens; D, digestive; EW, egg white FA, other food allergies; NA, not applicable; S, skin; SBFC: single blind food challenge

Egg OIT protocol was performed with commercial pasteurized liquid EW (8.3% protein; Huevos Guillén, Valencia, Spain). As described in **Table 14** the highest tolerated dose during the SBFC was used as the starting dose for the OIT protocol. In this protocol, once the patients were able to tolerate 2 mL of undiluted EW without symptoms, an open challenge with baked goods containing egg was performed. Similarly, after tolerating 4 mL of EW, patients were challenged with egg coated foods, with boiled egg after 12 mL, French omelette after 20 mL and, finally, with a fried egg after 32 mL. Between hospital visits, patients were advised to daily ingest at home the maximum dose achieved during their last visit to the unit. Premedication with antihistamines was given to those patients that developed adverse reactions during the protocol in order to control the symptoms if needed.

In the case of moderate reactions, these were pharmacologically treated and the protocol was restarted on the following week at the previously tolerated dose. Hence, the length of the protocol underwent stepwise increases depending on the severity of the reactions experienced by the patient. In the case of repeated severe reactions (anaphylaxis) the desensitization protocol was interrupted. Patients were considered to have completed the OIT protocol if they were able to tolerate 32 mL of pasteurized EW (equivalent to a full egg white) in less than 24 months.

Once the patients completed the OIT protocol they maintained during 6 months a daily dose of 16 mL of pasteurized EW at home, except for two days per week, when the patients were advised to consume a complete egg (omelette, boiled or fried egg). If after 6 months the clinical tolerance was sustained, the patients were authorized to have a non-restricted diet with the recommendation to eat eggs 2-3 times per week.

Day	EW dilution	Dose (mL)	Challenge
1	1/10	0.1	Pasteurized EW
8		0.5	
15	Undiluted	0.1	Pasterurized EW
22		0.5	
29		1.0	
36		2.0	Baked goods containing egg
43		4.0	Egg-coated foods
50		8.0	
57		12.0	Boiled egg
64		16.0	
71		20.0	French omelette
78		24.0	
85		28.0	
92		32.0	Fried egg

Table 14. Egg oral desensitization protocol. Once the patients were able to tolerate 2 mL of undiluted EW without symptoms, an open challenge with egg-containing bakery was performed. Similarly, after tolerating 4 mL of EW, patients were challenged with egg-coated foods, with boiled egg after 12 mL, French omelette after 20 mL and, finally, with a fried egg after 32 mL.

Efficacy and safety of desensitization

As described in **Table 13**, 12 of the 20 patients enrolled in the study (60%) (patients 1 to 12) completed the protocol being able to tolerate 32 mL of pasteurized EW in a mean period of 11.75 months (Mean ± SEM= 11.75 ± 1.34) [interval: 6-20 months]. Although according the original schedule, desensitization was planning to be achieved in 3 months, dosing regimen was adapted to the patient's response having to be increased in, approximately, 9 months. 50% of this group required premedication with antihistamines, and 7 of them developed symptoms during the protocol. The most common symptoms were mouth itching and lip edema followed by abdominal pain and/or vomiting. After 24 months of treatment, four patients (20%) (patients 13 to 16) tolerated between 2.0-10 mL of pasteurized EW, being able to consume baked goods containing egg and egg-coated foods without having anaphylactic reactions. These patients were considered partially desensitized. Obviously, they had the highest number of adverse reactions during the protocol. In patients 17 to 19 the protocol was stopped after several attempts, due to unacceptable severe adverse effects including, among others, recurrent anaphylactic manifestations, uvula edema and severe atopic dermatitis. Finally, only one patient (number 20) dropped the study for parental decision.

To date, no adverse events have been reported by successfully desensitized children at 36-48 months after completed OIT protocol. Regarding children who were partially desensitized, three of the four forming this group have reached the maximum dose programmed through these months, whereas the remaining child continues with partial tolerance (9 mL), being able to eat baked good containing egg, egg-coated foods and boiled egg without developing negative symptoms. No changes have been reported in children who initially failed desensitization. Thus, to date, 75% of patients are on a free egg diet.

Baseline status of cow´s milk allergic vs. non-allergic children

Egg-allergic patients displayed significantly higher levels of OVA-, OM- and EW- sIgE than not allergic subjects, whose levels were almost zero (*P*<0.001) (**Figure 22A**). Regarding the allergen-sIgG4 levels, although not statistically significant differences, results showed a trend towards higher concentrations of OVA-sIgG4 (*P*=0.21), OM-sIgG4 (*P*=0.38) and EW-sIgG4 (*P*=0.09) in the non-allergic group when compared with the egg allergic group (**Figure 19B**).

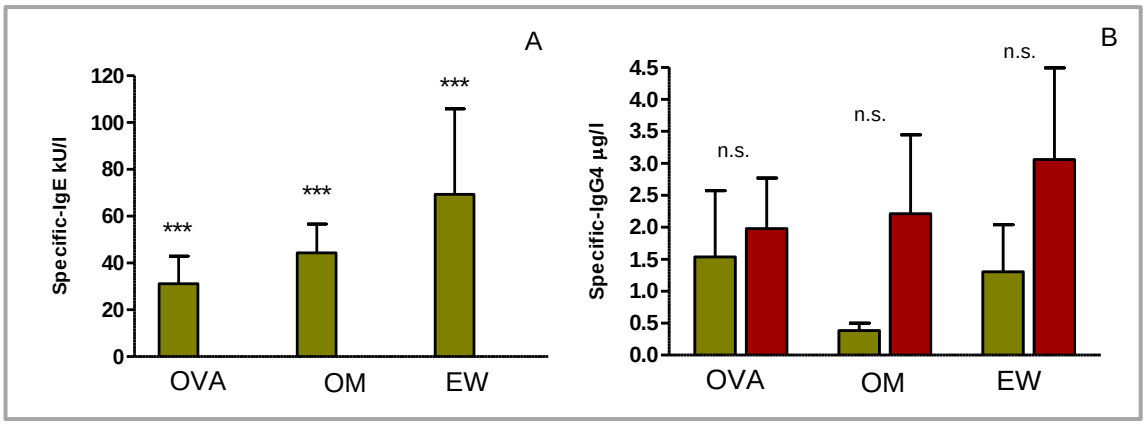

Figure 22. Serum specific (**A**) IgE and (**B**) IgG4 to OVA, OM and EW before the beginning of OIT in egg allergic children (N=20, *green bars*) and non-allergic controls (N=15, *red bars*). Bars represent mean ± SEM. Mann-Whitney 2-tailed test and 95% confidence intervals. ***: *P*< 0.001; n.s.: non-significant differences.

As presented in **Table 15**, although non-statistically significant, the mean production of Th2 cytokines (IL-5 and IL-13) by OVA-stimulated-PBMCs was higher in the egg allergic patients compared with non-allergic children. In contrast, a decreased Th1 cytokine concentration (significant for OVA-specific TNF-α) was found in this group of patients. A statistically significant higher production was found for OVA-specific IL-10 in non-allergic children compared with egg-allergic patients.

Cytokine	Allergic (N=20)	Non-allergic (N=9)	*P*-value
IL-5	38.49 ± 22.11	8.95± 4.15	0.26
IL-13	104.90 ± 35.00	57.27 ± 16.23	0.27
IFN-γ	22.60 ± 8.49	82.44 ± 34.71	0.11
TNF-α	96.90 ± 26.94	409.40 ± 161.20	**0.042**
IL-10	120.2 ± 19.16	337.60 ± 75.73	**0.007**

Table 15. Cytokine production by OVA-stimulated PBMCs from egg allergic children before OIT and from non-allergic children. Values are presented as mean ± SEM. *P*<0.05 by Mann-Whitney U-test (2-tailed) was considered significant.

Immunologic outcomes during OIT (Egg OIT outcomes)

***Egg OIT outcomes*: Specific antibody response**

All the patients that completed the egg OIT protocol (N=12) had a significant decrease (>5-fold), in their OVA-, OM- and EW-sIgE levels (**Figure 23A**) once the protocol was finished. Furthermore, a significant increase from baseline was observed in OVA-, OM- and EW-sIgG4 after the egg-OIT treatment (**Figure 23B**).

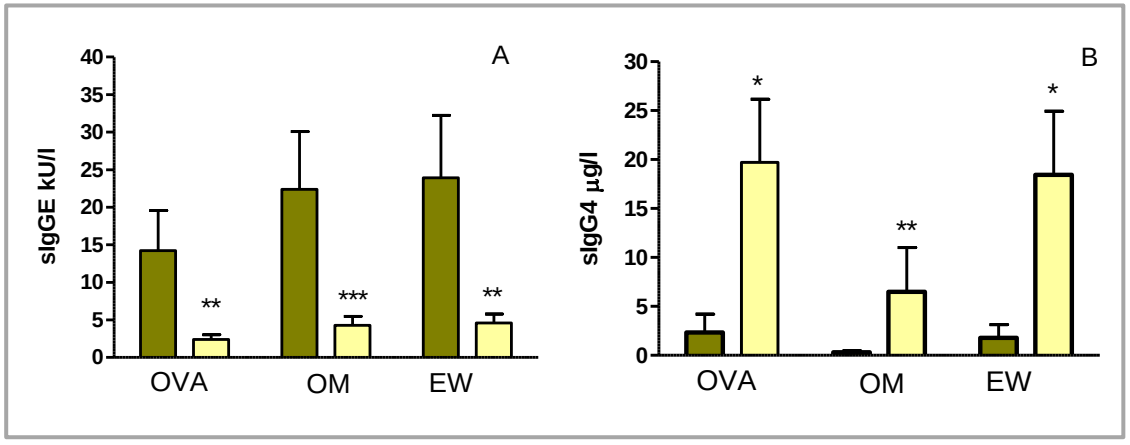

Figure 23. Serum specific (**A**) IgE and (**B**) IgG4 to OVA, OM and EW before (*green bars*) and after (*yellow bars*) the OIT protocol in patients who tolerated 32 mL of egg white (N=12). Bars represent mean ± SEM. Wilcoxon 2-tailed test and 95% confidence intervals. ***: *P* < 0.001, **: *P* < 0.01, *: *P* < 0.05

***Egg OIT outcomes*: OVA-specific cytokine production by PBMCs**

When comparing allergen-specific cytokine levels, before and after the egg OIT protocol in the group of patients who tolerated 32 mL of EW (N=12), it was found a trend towards a reduced IL-5 and IL-13 production by PBMCs after OVA stimulation, together with an increase in the concentrations of the Th1 cytokines IFN-γ and TNF-α. Furthermore, levels of OVA-specific IL10 were significantly higher when compared with baseline (*P*< 0.05) (**Table 16**).

Cytokine	Egg allergic patients who tolerated 32 mL of EW before and after OIT (N=12)			Egg allergic patients who did not successfully complete OIT (N=7)	
	Before OIT	After OIT	*P*-value [a]	Before OIT	*P*-value [b]
IL-5	11.93 ± 9.23	3.83 ± 3.22	0.42	89.51 ± 59.02	0.11
IL-13	79.15 ± 23.93	52.12 ± 21.53	0.41	163.70 ± 90.94	0.28
IFN-γ	24.50 ± 10.45	53.73 ± 19.33	0.20	22.41 ± 17.19	0.91
TNF-α	131.70 ± 39.76	277.10 ± 212.90	0.51	47.25 ± 26.88	0.15
IL-10	97.73 ± 24.03	195.1± 38.75	0.046	141.11 ± 30.39	0.29

Table 16. Cytokine levels after stimulation for 7 days with OVA of PBMCs from egg allergic patients who tolerated 32 mL of egg white before and after completing the egg OIT and baseline levels for egg allergic patients who did not successfully completed the protocol. Values are presented as mean ± SEM. *P*<0.05 by Mann-Whitney 2-tailed test was considered significant.

[a] Before *vs*. after OIT in patients who completed the protocol

[b] Before OIT in patients who completed the protocol *vs*. patients who did not complete the protocol

***Egg OIT outcomes*: Gene expression in OVA-stimulated PBMCs**

Transcription factors FoxP3, Tbet and GATA3 were upregulated in more than 66% of the desensitized patients. However, the expression revealed no enough variation to consider significant changes as mean RQ values were very close to 1 (Mean ± SEM [range] FoxP3 = 1.57 ± 0.22 [0.55-2.83]; T-bet = 1.76 ± 0.28 [0.28-3.93]), GATA3= 1.19 ± 0.14 [0.29-2.06]) (**Figure 24**).

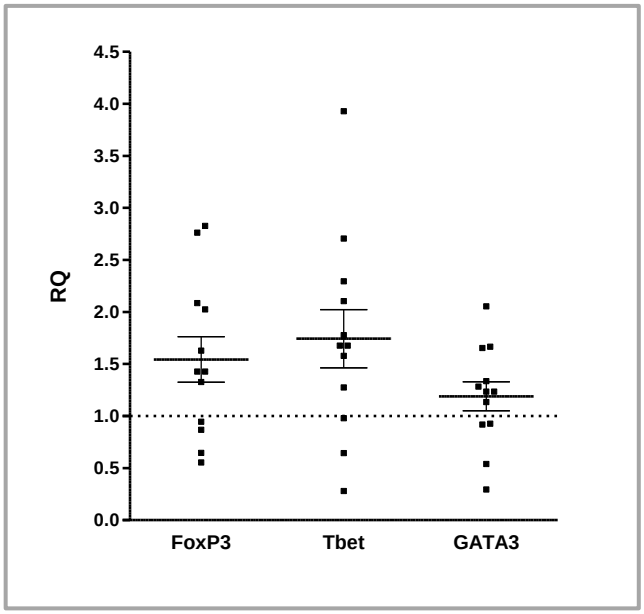

Figure 24. RQ values of transcription factors in response to intervention in complete number of egg allergy patients completed OIT (N=12). Black square represents change from baseline to successful OIT. RQ value of 1 means no difference in two times.

Baseline immunological status of fully desensitized egg allergic patients vs. partially desensitized

When baseline OVA, OM and EW sIgE levels were compared between the patients successfully desensitized to egg (n=12) and those that did not fully complete the protocol (N=7), it was found that egg-protein sIgE levels were higher in the latter (Mean ± SEM for OVA-sIgE: 14.23 ± 2.51 *vs* 62.98 ± 29.89 (*P*=0.047); Mean ± SEM for OM-sIgE: 22.39 ± 7.68 *vs* 82.53 ± 28.33 (*P*=0.07); Mean ± SEM for EW-sIgE: 23.93 ± 8.30 *vs* 153.30 ± 99.88 (*P*=0.07)).

Regarding the baseline production of cytokines by OVA-stimulated PBMCs, it was observed lower mean values of IL-5 and IL-13, together with higher TNF-α mean level in the patients who successfully completed the protocol, although they were non-statistically significant changes (**Table 16**).

4.2.3.2. DISCUSSION

In the current study, the immunological status of the enrolled egg-allergic children was initially assessed, and compared with that of a group of non-allergic patients from the same age-range. In addition to the presence of egg protein-sIgE antibodies in plasma, characteristic of the allergic status (Burks et al., 2012), PBMCs from egg-allergic patients showed a diminished OVA-specific TNF-α and IL-10 production, together with a trend towards higher IL-5 and IL-13 levels upon OVA stimulation. These findings demonstrating a bias towards Th2 type cytokine production in food allergic individuals. We speculate that the decreased Th1 and IL-10 responses observed in allergic patients might represent a reduction in Th1 and IL-10 regulatory cell populations capable of suppressing Th2 responses. IL-10 particularly, is known to play an important role in Treg cells function (Palomares, 2013).

The efficiency and safety of the proposed egg OIT protocol were in the range of reported in studies by *Dello Iacono et al.* (2013) and *Meglio et al*. (2013), in which OIT led to the clinical desensitization of 90% and 80% of the children respectively. Due to moderate side effects, the actual duration of this desensitization protocol was much longer than originally expected, and had to be increased in, approximately, 9 months in relation to the original desensitization schedule. Similar results were observed in the course of the CM-OIT previously reported in this thesis (Section *4.1.1.1.2.)*. Something similar was reported in the study by *Staden et al*. (2007), where the length of the planned protocol was increased from 67 days to 7 months because of adverse reactions. Similarly, *Meglio et al*. (2013) increased their protocol in 150 days owing to intercurrent illnesses. *Narisety et al*. (2009) observed in their milk OIT study that prolonged higher-dose treatment induced new immunological changes and enhanced the desensitization effect, suggesting that longer treatment courses are more effective and possibly safer. In addition, results in *Vickery et al.* (2010) point out that desensitization periods cannot be planned in advance, making it necessary to individualize the dosing according to the patients characteristics.

A spontaneous outgrown of egg allergy in the patients included in the study was very unlikely due to their high average baseline EW-sIgE levels, exceeding by far the established thresholds for clinical reactivity (Sicherer et al., 2014), and their frequent adverse responses to the therapy. On the other hand, the current protocol cannot confirm whether the patients were tolerant to egg, as the egg intake was not withdrawn after the treatment because of ethical reasons. In a previous report by *Burks et al*. (2012) in which egg was avoided for a period of 4 to 6 weeks after a desensitization protocol, more than 70% of the patients initially desensitized did not pass a subsequent oral food challenge. Anyhow, it is worth to mention that all the patients that successfully finished the reported egg OIT protocol (12/20) were still able to tolerate a complete egg after passing 36-48 months of the therapy. Further, 3 out of 4 partially desensitized children have reached the maximum dose programmed (32 mL of EW) during this time. Thus, the desensitization rate increased to up to 75% patients (15/20).

The observed changes in antigen sIgG4 production in successful OIT subjects mirror those observed in other food OIT protocols where antigen sIgG4 increase at the end of the immunotherapy (Blumchen et al., 2010; Itoh et al., 2010, Vickery et al., 2010; Varshney et al., 2011; Burks et al., 2012, Meglio et al., 2013). Regarding antigen-sIgE levels, a significant reduction, in accordance with other authors (Itoh et al., 2010; Vickerey et al., 2010; García Rodríguez et al., 2011, Dello Iacono et al., 2013; Meglio et al., 2013; Vila et al., 2013), was observed in our study. However, other reports did not find significant changes in IgE levels along the OIT protocol (Buchanan et al., 2007; Blumchen et al., 2011; Burks et al., 2012; Fuentes-Aparicio et al., 2013). The decrease in the IgE/IgG4 ratio observed during the OIT might be a feature of skewing from allergen-specific Th2 to Treg cells predominance. This affirmation is supported by the significant increase in IL-10 production by OVA-stimulated PBMCs as well as the reduction in IL5 and IL13 levels, found in the patients able to ingest 32 mL of pasteurized EW without developing symptoms. Although in our study we have not measured the populations of egg-specific Treg cells, the role of this cell type in tolerance induction after immunotherapy treatments has been demonstrated (Varshney et al., 2011; Urra et al., 2012; Fuentes-Aparicio et al., 2014).

Tregs, trough IL-10 secretion, are potent suppressors of both total and allergen-sIgE, whereas they simultaneously increase IgG4 production. Gene expression outcomes showed a lack of treatment-related changes in the expression of Treg (Foxp3), Th1 (Tbet), or Th2 (GATA3) transcription factors, despite the existence of measurable variations in involved OIT transcripts was already reported by *Jones et al*. (2009) in their study with peanut oral immunotherapy.

In the present research, lower baseline antigen-sIgE levels appear to be related with a successful OIT therapy. A similar observation was made by *García-Rodríguez et al*. (2011) and *Vazquez-Ortiz et al*. (2014), who found in their studies that elevated baseline levels of egg-sIgE were related with a high probability of discontinuation of the protocol. Furthermore, although not significant, patients able to tolerate 32 mL of pasteurized EW displayed a trend towards lower baseline levels of Th2 cytokines, together with an increase in TNF-α secretion after OVA stimulation of PBMCs. In the same line, IL-4 mRNA has been proposed as a possible predictive factor of egg allergy resolution by *Sicherer et al*. (2014).

Taken altogether, this report presents an efficient and safe egg-OIT protocol characterized for the progressive introduction of egg containing foods, which improve substantially the patient's quality of life. Successful OIT was accompanied by a significant increase in egg-sIgG4 levels and IL-10 production by OVA stimulated PBMCs as well as by a significant reduction in egg-sIgE concentration towards a non-allergic phenotype. Results of the study highlight the importance of designing individualized protocols taking into account the baseline egg-sIgE levels of the patient in order to achieve a successful desensitization.

5. CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. - The establishment of cow's milk protein allergy in infants was related with a deficit in the number of circulating Treg, which seems to result in an inadequate control of effector T cells, triggering a higher frequency of IL-4 secreting CD4+ T cells. These immune alterations would be crucial factors behind the onset of the allergic process and they could constitute a therapeutic target for the treatment of this food allergy.

2. - Treg deficit in cow's milk protein allergic children was not due to a defect in the thymic production of Treg, but seems to be related with decreased serum levels of vitamin D. Statistical analysis indicates that Treg and vitamin D values could be good predictors to discriminate between healthy controls and cow's milk protein allergic children, and also to predict the spontaneous achievement of tolerance.

3. - Egg ROIT approach had a greater rate of success than longer protocol, leading children to clinical desensitization to egg proteins in a few days, with most adverse reactions being controlled and providing with long-term protection.

4. - Cow's milk and egg long-course OIT protocols, based on individualized up-dosing and characterized by the progressive introduction of different foods containing the implicated allergens, were successful to induce desensitization while patient’s quality of life improved substantially. The long-term efficacy of these protocols was also demonstrated.

5. - The cytokine profile after allergen-specific stimulation of PBMCs could be used to predict the allergic status of the OIT participants, as allergic children displayed a bias toward Th2 type cytokine production and decreased Th1 responses compared with non-allergic individuals.

6. - Successful cow's milk and egg OIT were accompanied by significant reductions in antigen-specific IgE levels, whereas antigen-specific IgG4 levels were increased. Patients

who completed desensitization also showed decreased Th2-cytokine related levels by allergen-stimulated PBMCs after treatment. No treatment-related changes were found in the gene expression of any studied transcription factors.

7. - Lower baseline antigen-specific IgE levels were suggested as predictors to accurately define the efficacy and the risk of adverse reactions during egg OIT. High baseline CN-sIgG4 levels seemed to have important biological significance in cow's milk OIT and could be proposed as predictors of negative clinical response to therapy. A decrease in OVA-specific IL-13 at short time of immunotherapy could be a useful biomarker of positive response to egg in rush protocols of egg OIT.

CONCLUSIONES

1.- El establecimiento de la alergia a proteínas de la leche de vaca en lactantes se relacionó con un déficit en el número de Treg circulantes, lo que parece conducir a un control inadecuado de las células T efectoras y aumentando la frecuencia de células T CD4+ secretoras de IL-4. Estas alteraciones inmunitarias serían factores cruciales en el inicio del proceso alérgico y podrían constituir una posible diana terapéutica para el tratamiento de esta alergia alimentaria.

2.- El déficit de Treg en los niños alérgicos a proteínas de leche de vaca no se debió a un defecto en la producción tímica de Treg, sino que estuvo relacionado con una disminución de los niveles séricos de vitamina D. El análisis estadístico indica que los valores de Treg y vitamina D podrían ser buenos predictores para discriminar entre individuos sanos y los niños alérgicos a proteínas de la leche de vaca, y también serían de utilidad para predecir el desarrollo espontáneo de tolerancia.

3.- La ITO rápida al huevo tuvo una mayor tasa de éxito que el protocolo de larga duración, llevando a la desensibilización a las proteínas del huevo en pocos días. En estos pacientes, la mayor parte de las reacciones adversas fueron controladas y se proporcionó a los niños una protección a largo plazo.

4.- Los protocolos de ITO de larga duración a la leche de vaca y al huevo, basados en el aumento individualizado de la dosis y caracterizados por la introducción progresiva de diferentes alimentos conteniendo los alérgenos implicados, se mostraron exitosos en la inducción de desensibilización, conduciendo a mejoras sustanciales en la calidad de vida del paciente. La eficacia a largo plazo de estos protocolos ha sido también demostrada.

5.- El perfil de citoquinas secretadas tras la estimulación alérgeno-específica de PBMCs podría ser utilizado para predecir el estado alérgico de los participantes en la ITO, al

mostrar los niños alérgicos un sesgo hacia la producción de citoquinas de tipo Th2 con disminución de las respuestas Th1 en comparación con los individuos no alérgicos.

6.- La ITO exitosa a la leche de vaca y al huevo se acompañó de reducciones significativas en los niveles de IgE específica a alérgenos, mientras que los niveles de IgG4 alérgeno-específicos aumentaron. Los pacientes que alcanzaron la desensibilización también mostraron una disminución en los niveles de citoquinas Th2 secretadas por PBMCs estimuladas con alérgenos, una vez finalizado el tratamiento. No se encontraron cambios significativos en la expresión génica en ninguno de los factores de transcripción estudiados.

7.- Niveles basales más bajos de IgE específica a alérgenos podrían servir como predictores a la hora de definir con mayor precisión la eficacia potencial y el riesgo de reacciones adversas durante los protocolos de ITO al huevo. Los niveles basales más elevados de IgG4 específica a caseína parecen tener un significado biológico importante en la ITO a la leche de vaca, por lo que podrían ser propuestos como posibles predictores de una respuesta clínica al tratamiento negativa. La disminución de IL-13 específica a OVA al poco tiempo de tratamiento podría ser un biomarcador útil de respuesta positiva en los protocolos de ITO rápida al huevo.

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