



Immunotherapy in allergic diseases — improved understanding and innovation for enhanced effectiveness

Dimitrios I Mitsias¹, Paraskevi Xepapadaki¹, Michael Makris² and Nikolaos G Papadopoulos^{1,3}

Allergen immunotherapy leads to tolerance through multiple mechanisms that include tolerogenic dendritic cells and T and B regulatory cells. These induced cellular populations produce mediators to skew the immune response to a tolerogenic milieu that, among others, results in IgG4 blocking antibodies formation and lowered Fcε receptors. All lead in decreased effector responses from mast cells, eosinophils, and basophils thus limiting the allergic inflammation. Clinically, this results in better allergic rhinitis control and, of importance, asthma prevention. Newer approaches include modified allergens, second generation adjuvants/carriers and routes of administration, all aiming to increased efficacy with parallel no compromise of safety.

Addresses

¹ Allergy Department, 2nd Pediatric Clinic, National and Kapodistrian University of Athens, Thivon and Levadias 1, 115 27, Athens, Greece

² Allergy Unit, 2nd Department of Dermatology and Venereology, 'Attikon' University Hospital, National and Kapodistrian University of Athens, Rimini 1, 124 62, Athens, Greece

³ Division of Infection, Immunity & Respiratory Medicine, Royal Manchester Children's Hospital, University of Manchester, Oxford Rd., M13 9PL, Manchester, UK

Corresponding author:

Papadopoulos, Nikolaos G (nikolaos.papadopoulos@manchester.ac.uk)

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Introduction

Allergen immunotherapy (AIT) aspires to be the only etiologic treatment for respiratory allergies [allergic rhinitis (AR) and asthma] and may modify the natural history of the disease. Multiple mechanisms underpinning AIT have been identified, reflecting the various levels of allergic inflammation active in respiratory allergies. Improved understanding facilitates new approaches

designed to improve both efficacy and safety of AIT, as well as specificity and targeting. Herein, we review recent efforts and upcoming modalities for AIT improvement for respiratory allergies, in parallel to the exploration of immune mechanisms. Oral immunotherapy for food allergy, albeit field of extensive research, was out of the scope of the current review.

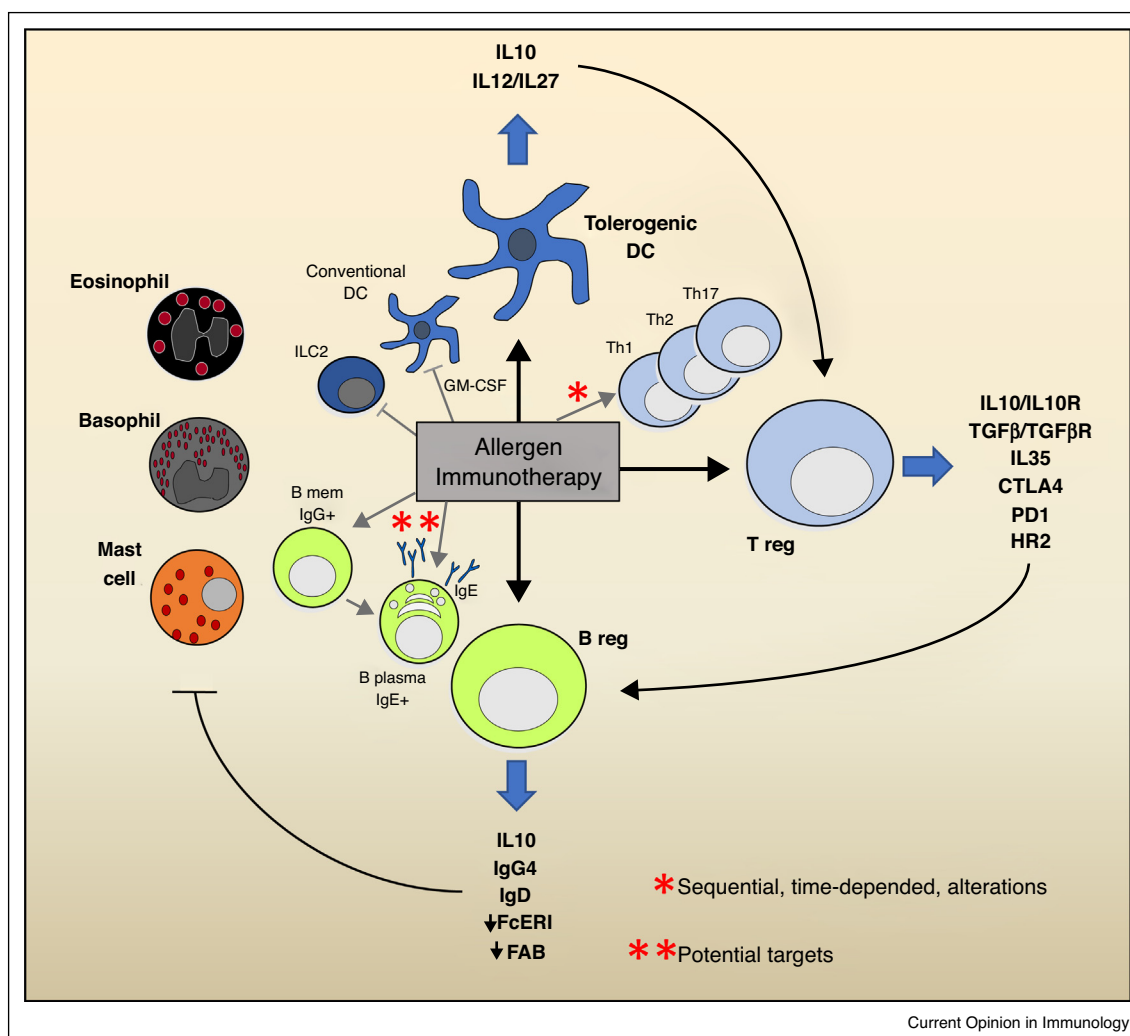
Key mechanisms of allergen immunotherapy

The induction of regulatory T cells (Treg) following AIT, initially reported almost 15 years ago, remains central in our understanding of the immune mechanisms of AIT. It has become clear that these cells exert pluripotent actions via multiple suppressor factors including IL10, TGFβ, IL35, CTLA4, IL10R, TGFβR, PD1, and HR2; thus, controlling the pro-allergic milieu. Tolerogenic dendritic cells (DCs) are thus restored, promoting T cell tolerance followed by the suppression of the effector cells of allergic inflammation — mast cells, basophils, and eosinophils. Furthermore, Treg promote the induction of regulatory B cells (Breg) inducing the secretion of allergen neutralizing IgG4 antibodies that further diminish FcεRI and CD23-mediated allergic responses [1] (Figure 1).

Several clinical studies, including a recent one evaluating subcutaneous (SCIT) and sublingual (SLIT) immunotherapy in grass-allergic patients [2], have shown that AIT induces the production of both peripheral and local functional IgG4 antibodies. Despite the reduction of clinical efficacy after the 2-year treatment period, IgG4 levels and IgE-Facilitated Allergen Binding (IgE-FAB) to B-cells persisted through the 3rd year. The clinical outcome as addressed by symptom scores correlated more consistently with the local inhibitory activity of IgG4 antibodies against IgE-FAB to B cells.

In the Prospective Allergy and Clinical Immune Function Cohort study (PACIFIC) in grass pollen allergic patients undergoing AIT, three distinct phases in the hierarchy of tolerance were described: the up dosing phase, marked by increased IL-10⁺ B-cells with allergen-specific PD-L1 upregulation, while effector Th1, Th17 cells, and CCR6⁺IL-17⁺FoxP3⁺T-cells (Treg) decrease. The subsequent, conversion phase exhibits Th17 recovery in the absence of Th2 cells. Finally, the tolerance-mounting

Figure 1



Suggested mechanisms in allergen immunotherapy discussed in the text. Simplified review of current and upcoming knowledge on the impact of AIT on key cell populations, that is, dendritic cells (DC)/intraepithelial lymphatic cells 2 (ILC2), T cells, B cells, and effector cells (mast cells, eosinophils and basophils), as well as humoral mediators.

phase, after three years of treatment, was characterized by induction of Treg with simultaneous suppression of Th2 and allergen-specific Th17 responses. Notably, high ratios of circulating Breg/Th17 following initial AIT correlate significantly with clinical improvement after three years [3^{••}].

A time-course SLIT study, combining VH repertoire sequencing and single cell transcriptomics yielded direct evidence of a parallel boost of two clonally and functionally related B-cell subsets, short-lived IgE + plasmablasts and IgG + memory B cells. Mucosal grass pollen allergen exposure by SLIT resulted in highly diverse IgE and IgG + memory B-cells repertoires. These were extensively mutated and appeared relative stable as per heavy chain

isotype, somatic hypermutations and clonal composition. The stable IgE repertoire during the first year of SLIT provides evidence to why long-term AIT is not associated with disease progression [4^{••}].

Disease modification through AIT

Early onset asthma is largely associated with IgE sensitization [5]. Therefore, AIT has long been suggested as a disease modifying strategy potentially preventing allergen sensitization and asthma occurrence [6]. Allergen sensitization is considered a precursor of later clinically related airway diseases [7]. In a recent murine model, inhalation of a major HDM allergen prevented the onset of HDM sensitization and HDM-induced asthma by suppressing lung Granulocyte-Macrophage Colony-Stimulating Factor

(GM-CSF)-driven activation of type 2 conventional DCs [8]. Although early clinical studies showed a preventive effect of AIT on the occurrence of new sensitizations in children with rhinitis [9], this is still inconclusive [10]. Early aeroallergen exposure through AIT in high risk children had no effect on later allergy prevention in a pilot study [11]. A recent meta-analysis identified only a short-term preventive effect of AIT on novel sensitization development; nevertheless, the included studies were highly heterogeneous [12]. In a proof-of-concept study, in high-risk 5–9 month infants treated with HDM-SLIT extract, no effect on HDM sensitization or any clinical allergy-related outcomes; that is, eczema, wheeze, and food allergy, was noted, despite a significant reduction on cumulative sensitizations to common aero and food allergens (HDM, grass pollen, cat, peanut, milk, and egg), as assessed by skin prick tests [13]. In a pilot study including preschool aged children, mono/oligosensitized to HDM or grass and treated with SLIT for two years, although a significant induction of allergen-specific IgG4 and IL-10 was shown, the sensitization preventive effect of SLIT could not be documented [14].

AIT has been assessed in children with AR in order to prevent asthma. The preventive effects of AIT on asthma development and progression are confirmed from real-world studies assessing the effect of immunotherapy in subjects with allergic rhinitis [15,16]. Using robust controlled methodology to avoid past shortcomings [17], the Grazax Asthma Prevention (GAP) study showed significantly lower and long-lasting incidence of asthma following 3-year AIT in grass AR subjects, while significant lower levels of total and allergen-specific IgE and reductions in skin prick test response to grass were noted [18].

A number of immunological changes occur in the course of AIT, associated with tolerance induction and potentially disease modification [19]. An increased abundance of active antigen-specific Breg and Treg cells is strongly associated with clinical tolerance [20,21] and selective deletion of epitope-specific Th2 cells affecting Th2 cytokine production, which in turn further restores the number of Breg/Treg and immunoglobulin isotype levels, is further suggested [22]. Upregulated antigen-specific regulatory cells and monocyte-derived macrophages contribute to enhanced and long-term IL-10 production [20]. IL-10 suppresses Th2 responses, inhibits pro-inflammatory responses and DCs required for the activation of effector T cells, and increases antigen-specific IgG4 production, which in turn competitively inhibits IgE binding on the surface of effector cells thus preventing degranulation, downregulates M2 macrophages and increases IL10, IL6, TNF α and CCL1 production [23]. Several tolerogenic factors such as IL35, IL1 receptor antagonist and TGF β , derive from Treg and Breg cells, while surface molecules such as CTLA4 and PD1, induce B cell switching, thus further decreasing IgE production and enhancing

IgG4, IgG2 and IgA2 production [24]. Recently, upregulation of allergen-specific IgD levels during AIT was significantly associated with markers of asthma control thus being proposed as novel marker for tolerance induction during AIT [25]. In respect to lung immunological mechanisms, AIT was shown to suppress both the eosinophilic and Th2-mediated inflammatory responses in an experimental model [26]. From another perspective, airway hyper-responsiveness, a hallmark of allergic asthma, is significantly reduced after AIT, possibly due to down-regulation of endothelin-1, a potent bronchoconstrictor, or CD4 $^{+}$ T cell shift to an 'anergic' Th2 phenotype [26,27].

Combining immunotherapy with biologics

The introduction of biologics in allergy therapeutics presents new opportunities in combining these with AIT, not only improving patient management, but possibly improving understanding of the immune pathways involved. Two distinct rationales have/are been/being exploited. The first involves blocking of effector mechanisms to reduce AIT adverse events and allow time for desensitization. The second suggests that immune pathway modifications by either anti-IgE or anti-IL4 may be able to promote the tolerogenic effect of AIT.

IgE blockade with omalizumab has been used for over 15 years against severe allergic asthma and, more recently, idiopathic urticaria. Interestingly, blocking circulating IgE does not only prevent mast cell degranulation, but also downregulates Fc ϵ RI, potentially suppressing antigen presentation and induces Treg and Ig class switching from IgE to IgG4 [28]. A small number of clinical studies combining omalizumab with different types of immunotherapy have, in the most part, confirmed the potential for adverse event reduction through IgE blockade [29]; however, additional immunomodulation, or disease modification, remains uncertain.

Already in 2007, Klunker *et al.* evaluated CD23-dependent IgE-FAB to B-cells after ragweed AIT alone or in combination with omalizumab [30]. AIT alone partially inhibited allergen-IgE binding, while anti-IgE induced complete inhibition. Nevertheless, allergen-specific IgG4 levels increased only after AIT. More recent studies have focused in food allergy oral immunotherapy.

The IL4 pathway is another promising candidate. In one published study, the addition of an anti-IL4 antibody (VAK694) to SCIT did not confer additional benefit in suppressing the allergen-induced skin late-phase response [31]. Dupilumab is currently approved for severe atopic dermatitis and severe asthma, with several ongoing studies exploring additional indications such as chronic rhinosinusitis and eosinophil esophagitis. It targets the IL4 receptor α -chain, thus blocking IL4 and IL13 and affecting several type 2-associated pathways, from IgE production by B-cells, Th2 differentiation and intraepithelial lymphatic cells-2

(ILC2) induction via eosinophils and basophils [32]. There are four ongoing studies combining dupilumab with immunotherapy, reported in clinicaltrials.gov (NCT03558997, NCT03682770, NCT04148352, NCT03679676), only the first of which however targets a respiratory allergen.

New hypoallergenic compounds, adjuvants and carriers

While natural allergen extracts remain the mainstay for AIT, modified preparations such as allergoids increase safety [33]. Allergoids lack the allergenic conformational IgE epitopes while maintain the linear, immunogenic ones. Further treatment with glutaraldehyde and formaldehyde leads to high-molecular-weight polymers through intermolecular and intramolecular cross-linking, further increasing safety and efficacy [34,35].

Recombinant allergens, hypoallergenic derivatives and peptides (reviewed in Ref. [36]) have been studied with mixed results. These approaches still have not found their place in the market; however, the prospect of delivering large amounts of immunogenic, non-contaminated, lab-produced specific components is highly promising. An important challenge remains: the complex sensitization patterns of each patient, needing a precision approach that would probably call for delivery of all clinically relevant epitopes to each patient. Finally, before marketing, complex regulatory issues regarding the use of such products will have to be fully addressed [37].

Adjuvants are very often used to increase immunogenicity, as they seem to be advantageous over 'simple' allergen delivery [38]. From the first-generation traditional adjuvants, aluminum remains the most commonly used; its adsorbent properties allow slow allergen release, improve vaccine tolerability and promote uptake to immune cells. Calcium phosphate and microcrystalline tyrosine are also currently used while MPL (monophosphoryl lipid A) coupled to allergoids represents a newer approach with positive outcomes [39].

CpG oligonucleotides (CpG-ODN) bind TLR-9, increase IL-12, IL-10, and IL-22 and decrease TSLP, all leading to Th2 suppression. Both soluble and nanoparticle (NP) bound CpG-ODN have been tested, with the latter offering important advantages like better targeting, need for less dose and possible co-administration with allergens [40,41].

Virus-like particles (VLPs) are composed of proteins that form the viral capsid and are used as platforms for immune recognition, uptake and processing. Initially, 'allergen-free' VLPs were coupled to CpG-ODN or, interestingly, Th2 cytokines (such as IL4, IL5, and IL13) leading to IgG or IgA cytokine-neutralizing auto-antibodies, depending on site of application. Lately, VLPs coupled to allergens (e.g. Fel d1 and Art v1) have

been studied in mouse models showing Th1/Treg-dominated cytokine responses and increased Foxp3+ Treg numbers in the lungs [42,43].

Carbohydrate particles (CBPs) have been used in conjunction with Phl p5 and Fel d1 to prolong allergen exposure (depot effect probably greater than aluminum) and induce T-cell anergy and better antibody responses [44].

The PreS domain, part of the Hepatitis-B virus envelope, is highly immunogenic. Fusion proteins of PreS with allergens (e.g. Fel d1, Der p23, and Bet v1) had decreased allergenicity and increased immunogenicity, while studies with B-cell epitope peptides from the major timothy pollen allergens (Phl p1, 2, 5, & 6) have shown clinical effectiveness associated with production of allergen-specific IgG4 antibodies and reduction in allergen-specific T-cell proliferation by inhibition of IgE-FAB [45]. Tetanus toxoid is another adjuvant which, coupled to Der p1, showed immunogenicity with reduced IgE-reactivity [46].

Various approaches address the delivery of the allergenic/immunogenic compound to target cells: in a mouse model of AR, SLIT with liposomes carrying α -galactosylceramide [α -GalCer, a lipid antigen and potent activator of invariant natural killer T-cells (iNKT)] plus allergen was more effective compared to allergen alone [47]. Recently, liposomes were used to co-localize allergen and CD33L that binds the inhibitory receptor CD33 on mast cells [48]. Such constructs desensitize mast cells allowing the administration of AIT without the risk of IgE-mediated side effects.

Poly(D-L-lactide-co-glycolide) acid (PLGA) particles coated with *Vibrio cholerae* neuraminidase target M-cells in gastrointestinal mucosa and can augment allergen delivery in case of oral immunotherapy as shown earlier with an *Aleuria Aurantia* lectin (AAL)-coated PLGA particles carrying birch pollen [49]. Proteoliposomes of *Neisseria meningitidis* serogroup B have also been used as adjuvants for an HDM-AIT model [50].

Polymerized allergoids coupled to non-oxidized mannan delivered sublingually showed better Th1/Treg responses compared to native allergens. Oral DCs bind mannan through C-type lectins and therefore increase the uptake of the allergoids [51]. Similarly, antigen presenting cell (APC) uptake was enhanced when Fel d1 was fused to an MHC-II targeting domain along with a trans-activator of transcription (TAT)-peptide of HIV to increase pass through the APC cell membrane [52].

Despite the promising results of different adjuvants/carriers in animal models, human studies are few and eagerly expected.

Table 1***In vitro* Biomarkers of Allergen Immunotherapy [63,64]**

Domains	Biomarker	Pro	Con
IgE	IgE (sIgE, total IgE, sIgE/total IgE)	easy to measure, reproducible, gold standard for AIT selection	No correlation with good clinical outcome
IgG	sIgG4, sIgG1	serum measurement, 10-fold to 100-fold ↑sIgG4 by AIT	No consistent correlation with clinical response
Serum Inhibitory activity for IgE	IgE-FAB, IgE-BF	serum-based, reproducible	Technical complexity, confined to specialized laboratories, modest correlation with clinical response
Basophil activation	CD63 CD203c DAO	small amount of blood	Not standardized method
Cytokines/chemokines	Th2: IL4, IL13, IL9, IL17, eotaxin, TNF α Th1: IFN γ , IL12 Regulatory: IL10, TGF β	miniaturized multiplex analysis	Low sensitivity, unknown clinical relevance
Cellular biomarkers	Treg cells, Breg cells, DCs	distinguish between treatment groups, correlate overall with clinical outcomes	Unable to distinguish responders from non-responders and to predict response in individual subjects

Abbreviations: sIgE, specific IgE; sIgG4, specific IgG4, sIgG1, specific IgG1; IgE-FAB, IgE-facilitated allergen binding; IgE-BF, IgE-blocking factor; BHR, basophil histamine release; DAO, diamine oxidase; Treg, regulatory T cell; Breg, regulatory B cell; DC, dendritic cell.

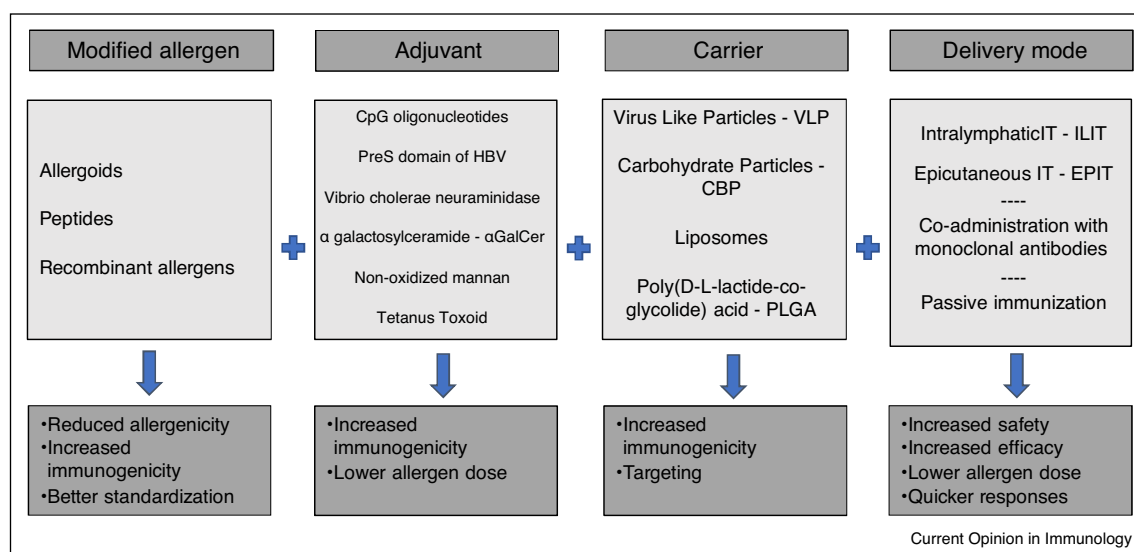
Routes of administration

For the moment, SCIT and SLIT are the main AIT routes of administration, lasting 3–5 years. Intralymphatic AIT administration [52^{••},53] is a very promising alternative since it is expected to be faster acting and uses less allergen; therefore, it may have higher acceptance with less side effects. Convenience and safety are also potential benefits of epicutaneous AIT [54,55], a route mainly studied for oral allergen immunotherapy in case of food

allergies. Passive immunization with monoclonal antibodies [56^{••}] seems to be an effective and safe alternative which possibly lacks, however, the disease modifying properties of classic AIT.

Biomarkers

Several assays assessing Th1 and Treg as well as cytokines released from these cells that are thought to drive

Figure 2

Schematic review of possible future candidates for more effective and safer immunotherapy. Each factor is expected to act either alone or in conjunction with any other.

immune tolerance, are been evaluated as biomarkers [57,58] (Table 1). The predictive value, specificity and sensitivity of these markers are not well determined; their use, although promising, needs further elucidation. The combined use of these biomarkers can only be done in clinical trials and specialized centers [59]. *In vivo* biomarkers including exhaled breath analysis [60] and allergen provocation studies especially in environmental exposure chambers (EECs) [61] are also in use mainly in clinical trials; standardization and protocol harmonization will improve the qualimeters (EECs) [61] are also in use mainly in clinical trials; standardization and protocol harmonization will improve the quality of data and provide clinically and immunologically relevant information.

New technologies as single-cell transcriptomics allow high resolution analyses of rare cells while the advances of multi-parameter flow cytometry and mass cytometry combined with proteomic approaches allow the study of proteins in solution, as well as from cell fractions [62]. Immunoglobulin and cytokine transcripts show promise for identification of molecular biomarkers for AIT success. However, simplified versions will have to be developed, allowing their implementation in clinical practice.

Conclusion

AIT remains highly promising; important advances in the understanding of its mechanisms, as well as novel approaches, including new adjuvants, improved biomarkers, combination with biologics, better-characterized allergens, and new administration routes are underway (Figure 2). However, these are slowed down by the regulatory requirement of safe and well-characterized products with general utility for patients with highly variable sensitization patterns and immune background. In addition to more studies, a novel paradigm for effective translation and commercialization is clearly needed.

Declaration of interests

Dr Mitsias reports personal fees from Novartis, Astra Zeneca, L'Oreal, Menarini and Gerolymatos Int., all outside the submitted work.

Dr Xepapadaki reports personal fees from Nutricia, Nestle, Friesland, Uriach, Novartis, Pharma AG, Glaxo Smith Kline, outside the submitted work.

Dr Makris declares personal fees from Novartis Hellas, GSK, Menarini, Sanofi, MYLAN, AstraZeneca, ELPEN, SARANTIS, GALENICA outside the submitted work.

Dr Papadopoulos reports personal fees from Novartis, Nutricia, HAL, Menarini/FAES FARMA, Sanofi, MYLAN, AstraZeneca, GSK, MSD, ASIT Biotech,

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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