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Biological and clinical significance of T helper 17 cell deficiency: insight into monogenic defects

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Summary

*T helper 17 (Th17) are a CD4+ T subpopulation cells which are involved in the
host protection against microbes such as extracellular and intracellular bacteria,
parasites, fungi, and viruses. Monogenic defects including those mutations in some
genes such as the signal transducer and activator of transcription (STAT)1 and 3,
dedicator of cytokinesis 8 (DOCK8), autoimmune regulator (AIRE), and inter-
leukin 17 receptor A (IL-17RA) can lead to impairment in Th17 cell development
and function along with the concomitant increased risk for chronic mucocutane-
ous candidiasis (CMC). The immunologic abnormalities in these patients include
low frequency of Th17 cells; defective cutaneous or in vitro T cell response to Can-
dida species, and/or autoantibodies against relevant cytokines. This review outlines
the biological characteristics and functionality of Th17 cells, as well as the clinical
features of individuals with genetic defects associated with Th17 deficiency.*

Introduction

T helper (Th) cells that synthesis Interleukin-17 (IL-17) are
derived from CD4+ T cells subpopulation which are associated
with protection of host against microbes including extracellular
bacteria and quite a number of fungal agents (1). Disease pro-
gression in various autoimmunity and inflammatory disorders
is due to direct involvement of Th17 cells which secret the IL-
17 family, cytokines including IL-17A and IL-17F, along with
IL-22 and granulocyte- macrophage colony-stimulating factor
(GM-CSF) (1). Cytokines like IL-17A and IL-17F often induce
neutrophil production and local recruitment by controlling the
expression of granulocyte-colony-stimulating factor (G-CSF)
and tissue expression of CXCR2 ligands such as CXCL8 (IL-8),
respectively (1). Neutrophils play a crucial role in the preven-
tion of invasive fungal infections such as those caused by *Can-
dida* (2, 3). Notably, Th17 cells analysis in human subjects and
various murine models studies have confirmed that Th17 cells
also play a pivotal function in mucosal immunity (1, 4).
Investigation on individuals that are prone to chronic mucocu-
taneous candidiasis (CMC) with defects in Th17 development
and functionality due to single nucleotide polymorphism or as
segment of syndrome have elucidated the involvement of Th17
cells in the protection of human host (5, 6). The processes of
Th17 cell differentiation and inborn errors of Th17 cell func-
tion in affected patients have drawn attention in recent time (7).
For instance, it has been reported that mutation in some genes
such as *DOCK8, STAT3, STAT1, AIRE, IL-17RA, IL-17F* led to
impairment of Th17 development and function in humans (8).

In this review, we summarized the biological characteristics and functions of Th17 cells as well as the clinical features of patients with genetic deficiencies associated with Th17 deficiency.

Biological characteristics and functions of Th17 cells

Th17 cells are described as producers of TNF-α and IL17, which represents a group of cytokines that are made up of IL17A-F, IL6, 21, 22, and 23 (9). Investigators revealed Th17 cells as one of the T-helper cells progeny having a distinctive host defense function against different extracellular infectious agents via effectors mediation of its secreted cytokines (**figure 1**). However,

Figure 1 - The biological functions of effector Th17 cells in human.
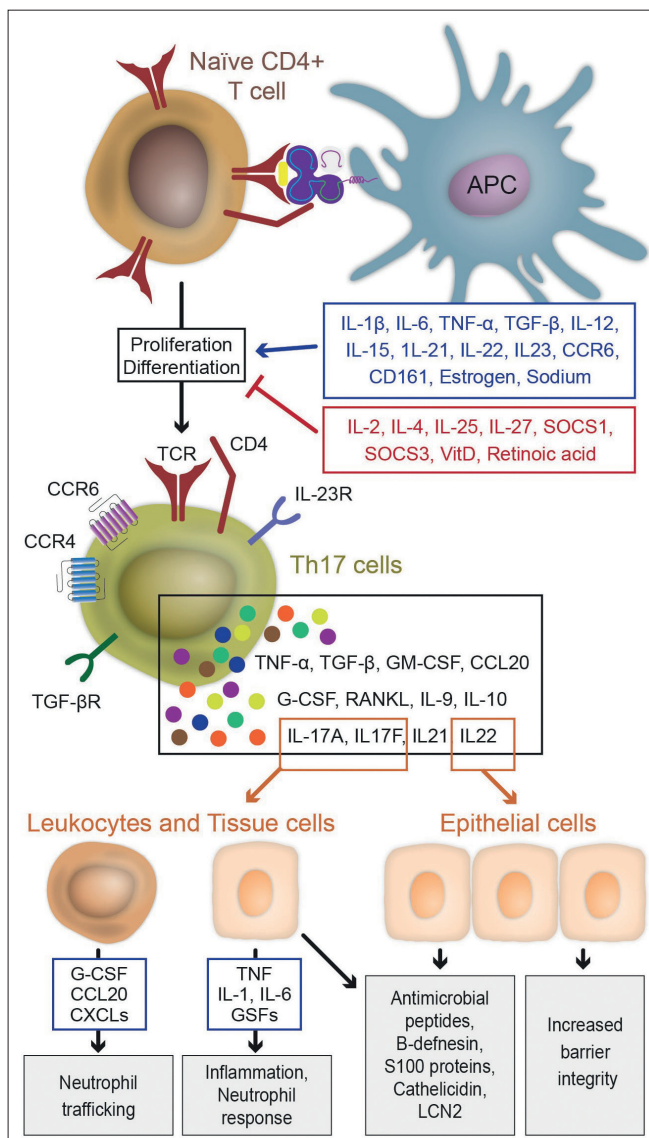
Th17 cells have been shown to be related with autoimmunity, carcinogenesis and certain chronic inflammatory diseases (10). Th17 cells and their associated cytokine IL-17 are responsible for various diseases such as psoriasis, asthma, tuberculosis and CMCC among many, but their precise roles in the pathogenesis of such diseases is not clearly understood. It was understood that IL-17 are associated with host defense against chronic inflammation, which also causes tissue damage or autoimmunity the activation of IL-17 signaling is via the IL-17 receptor binding by the IL-17 molecule, which in turn induces pro-inflammatory cytokines, neutrophil chemokines and antimicrobial peptides that are essential for antifungal activities (2). Since IL17 establishes a link between innate and adaptive immunity during the course of disease, it may have both physiological and pathological roles on host immune system. Evidence supports the notion that the dysregulated synthesis of IL17 and 21 by Th17 cells may contribute to the immunopathogenesis of autoimmune disorders (9).

Intracellular Bacteria

It has been studied that Th17 plays role in the immunogenicity of *Mycobacterium tuberculosis* but the precise role of its associated cytokine IL-17 in the infections is not clearly understood (11). In the early immune response to *M. tuberculosis* infection, Th17 cells stimulate recruitment of neutrophils, macrophages, and Th1 cells into the zone of inflammation while regulating the infection process as well (11). Th17 is also critical in the regulation of Th1 responses to *M. tuberculosis* infection as well (11). Nonetheless, insufficient Th17 cytokines appears unessential for protection against mycobacteria, and also in the lung, IL-17-producing cells were identified before the recruitment of IFN-γ-producing cells following antigen vaccination (11). Interestingly, for the protective recall response, IL-23 is required instead of IL-12 via the production of granulomas and initiation of activated CD4 T cells, which is as a result of IL-17 induced chemokine expression of CXCL19, CXCL10, and CXCL11, the recruitment of nuetrophils that facilitate the formation of granulomasis induced by CXCR3 ligand (11, 12). Consequently, humans that have been previously exposed to *M. tuberculosis* possessed IL-17- and IL-22-producing cells in peripheral blood mononuclear cells (PBMCs) that have been sensitized with *M. tuberculosis* antigens (Mtb-Ags). Nonetheless IL-17 and IL-22 are produced by the same Th17 cells in mice (13, 14). The search for IL-17 and IL-22 double producer cells was never a success in humans. But T-cells responsible for the production of IL-22 were frequently seen than those that produced IL-17 (15). Hence, these findings indicate that more studies should be conducted to reveal the precise mechanisms of actions of Th17/IL-17 in various *M. tuberculosis* strain infections or vaccinations.

Extracellular Bacteria

Host immune response due to Th17 cells in countering the pathogenic effect of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Helicobacter pylori*, and *Citrobacter rodentium*, has been investigated in different studies (16). In a research conducted by Aujla and colleagues highlighted some key findings on the IL-22 role in *K. pneumoniae* infection protection (17). Extracted tissue of *K. pneumoniae* infected mice demonstrated an IL-23 dependent IL-22 with relatively same kinetics as IL-17 and IL-17F (17).

The impact of both IL-17 and IL-22 in human bronchial epithelial (HBE) cells were revealed as well as off regulation of host defense genes (17) and that of IL-22 in epithelial cells regeneration post trauma (17).

The involvement of Th17 cells in patients infected with *Helicobacter pylori* (*H. pylori*) was also studied (17). A controlled analysis between the un-infected and infected biopsies of *H. pylori* revealed an increased concentration of IL-17 and IL-23 mRNA in the later. An increased in IL-17 was detected in both and CD4$^+$ cells and freshly obtained gastric lamina propria mononuclear cells (GLPMC), while only CD3$^+$ cells increase was detected in (GLPMC) and there is also increased levels of IFN-γ expression due to IL-23 on specific CD4 T cells (18).

Viruses

In an effort to delineate the role of IL-17 in viral pathogenesis, over-expression of IL-17 was identified as a factor (19, 20), while the research conducted by Smiley and his colleagues also demonstrated that a successfully immunized mice with rotavirus vaccine shows the production of protective IF-γ and IL-17 by Ag-specific CD4 T cells (21) despite the fact there is evidence of role of other factors in the protection against rotavirus because both IFNγ-R and IL-17R remain protected after successful immunization of KO mice (21).

Recently, an expression level of IL-17 and IL-22 was studied in HIV patient were increase concentration of IL-17$^+$CD3$^+$CD4$^+$ and IL-17$^+$CD3$^+$CD4 T cells peripheral blood in relation to HIV uninfected persons (22) while IL-22 and acute phase proteins high levels was seen in the studies by Misse *et al.*, due to activated T-cells in HIV infected and uninfected individuals (23).

Parasites

In an effort to study the role of Th17 in parasitic infection, mice with IL-17R deficiency and *Toxoplasma gondii* infected have shown relatively higher mortality, as there is low neutrophil recruitment in different tissues and CXCL8 expression, which resulted in higher parasitic burden (24). There is an increase in Th17 cells due to immunization with schistosome egg antigen in CFA (25) also IL-23p19$^{-/-}$ with severely reduced immunopathology, associated with reduced IL-17 producing T-cells in granulomas and a diminishing neutrophil recruitment due to reduced chemokine levels (26). To further explore the role of Th17 cells in parasitic immune response different experimental models need to be studied.

Fungi

Protective functionalities of Th17 in fungal immunity have been demonstrated in different instances. It was found that *Pneumocystis carinii* express IL-1β, IL-6 and IL-23. Inducing Th17 differentiation while blocking IL-23 or IL-17 by neutralizing antibodies significantly elevated the burden of *Pneumocystic* pneumonia. In addition, IL-23-deficient mice showed higher susceptibility to systemic *Cryptococcus neoformans* and pulmonary *P. carinii* infections (18, 19). Albicans candidiasis has also been shown to activate IL-23 expression in humans by memory T cells and monocyte-derived dendritic cells (DCs). These cells express CCR6 and CCR4 to produce IL-17 (20). Although, the IL-23/IL-17 pathway has been shown to enhance inflammation that inhibits the protective Th1 response against *Candida* and *Aspergillus* species (21, 22), Th17 cytokines and Th17 cells have been implicated in immunity against several infectious agents. For now, the origin of these cytokines in the innate host defenses is not fully understood. Therefore, there is the need for further investigations into the role of Th17 cells in chronic infection or memory responses in order to fully understand the innate host defenses against fungi.

Development and plasticity of Th17 cells

In 2008 Cosmi *et al.* showed that all Th17 cells originate from CD161$^+$ naive CD4$^+$ T cells and introduced CD161 as a surface marker for human Th17 cells (23). Studies by Bettelli *et al.* (24), Mangan *et al.* (25), and Veldhoen *et al.* (26) showed that IL-6, TGF-β, IL-1β and IL-23 were required for the induction of Th17 development. Despite these findings, the precise contribution of these cytokines in the development of human Th17 cells remains controversial (27). It has recently been revealed that naïve CD4$^+$T cells stimulated with TGF-β3/IL-6, or IL-1β/IL-6/IL-23, or TGF-β1/IL-6/IL-23 *in vitro* can differentiate to pathogenic Th17 cells and play important roles in the occurrence of autoimmune diseases. However, TGF-β1/IL-6-induced Th17 cells with suppressive function named by non-pathogenic or regulatory Th17 cells (28, 29).

In fact, the extremely dynamic process of Th17 subset differentiation shares an overlapping developmental axis with Th22, inducible regulatory T (iTreg) cells, and Th1 cells, which displays in an intermediary subsets of cells that display shared expression of lineage-specific transcription factors and cytokines (30).

Gut-associated lymphoid tissue (GALT) is a highly specialized region of the intestine where the differentiation of activated CD4+ T cells takes place, and provides additional layers of complexity towards modulating Th17 plasticity (30).

It was reported at least four major pathways and/or factors which contribute to Th17 plasticity (**figure 2**). The pro- and anti-inflammatory cytokine milieu directs CD4+ T cell development and modulates plasticity via the activation of specific signaling molecules and multiple transcription factors (30). Furthermore, it has emerged that immunoregulatory microRNA (miRNA) plays a critical role in controlling gene expression, thus influencing T cell fate and plasticity. It was reported that transcription factor aryl hydrocarbon receptor (AhR) and its physiological and environmental ligands alongside histone methylation and epigenetic modifications may influence T cell plasticity (30).

Th17 deficiency

Th17 deficiency due to defects in frequency and/or function of Th17 cells leads to unusual susceptibility to *C. albicans* infections (31). Some conditions that impair Th17 development and function in human are monogenic syndromes that prone patients to fungal infection including CMC (**table I**). The most known disorders are Hyper IgE syndrome (HIES) with auto-

Figure 2 - Development and plasticity of Th17 cells with other helper T subsets.
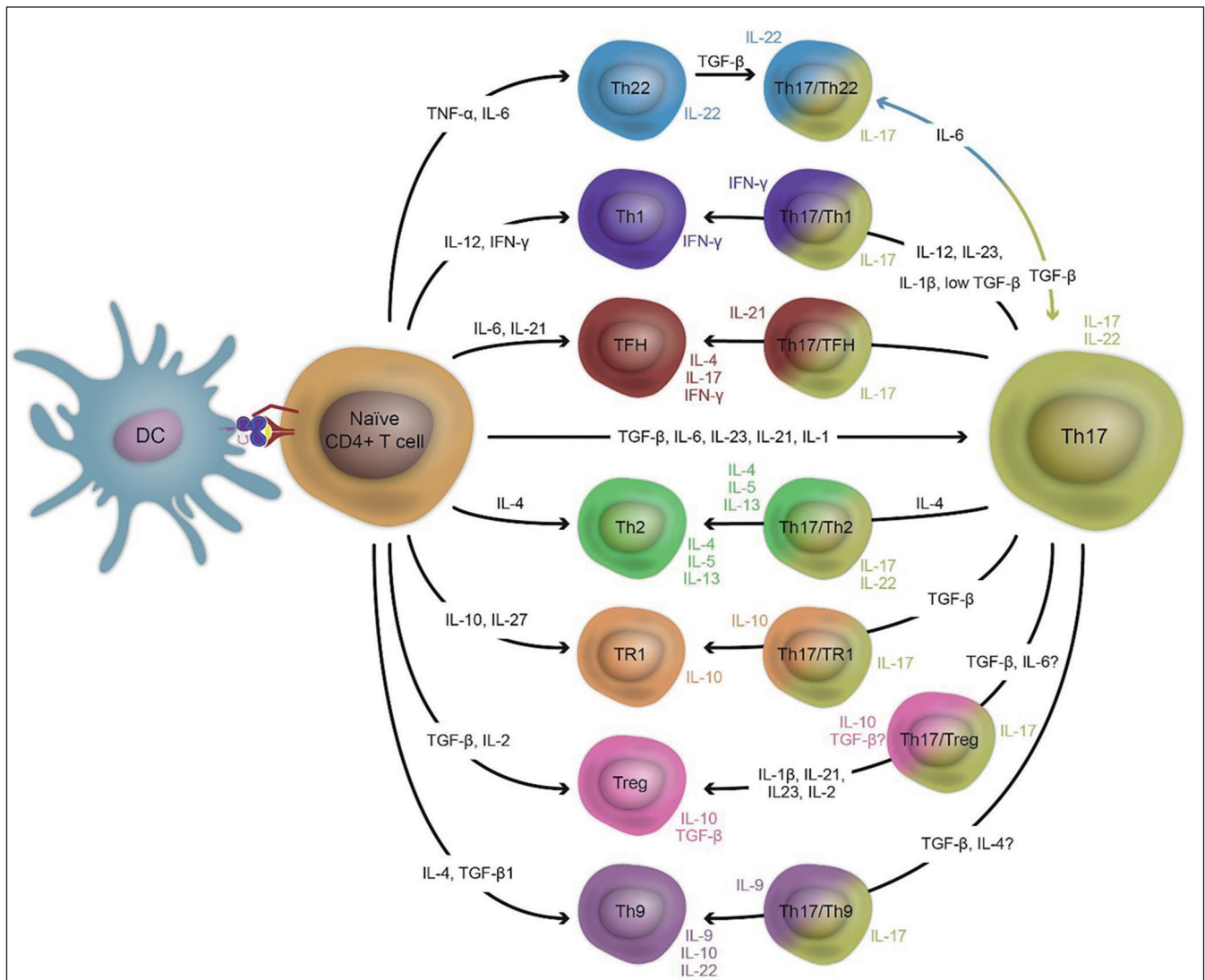
somal dominant and autosomal recessive inheritance**,** gain of function (GOF) mutation in STAT1, and APS-1/APECED syndrome (**figure 3**) (32, 33).

Monogenic defects leading to Th17 deficiency

Mutations in STAT1

A transcription factor called the signal transducer and activator of transcription 1 (STAT1) in humans is encoded by the *STAT1*

gene that regulates multiple biological downstream processes in a variety of cytokine receptors in many cell types (34). STAT1 mutations can be gain of function or loss of function (LOF) both of which can cause different phenotypes and symptoms. Recurring common infections are frequent in both GOF and LOF mutations. STAT1 GOF mutations, which account for approximately one-half of patients with CMC, was originally identified in a Ukrainian patient with CMC using whole-exome sequencing (35). Subsequently, mutations affecting STAT1's coiled-coil do-

Figure 3 - *Monogenic diseases that impair Th17 development or function against fungal pathogen. IL-1β, IL-6, IL-23, TNF-α and TGF-β are secreted after activation of antigen-presenting cells by fungal antigens.*
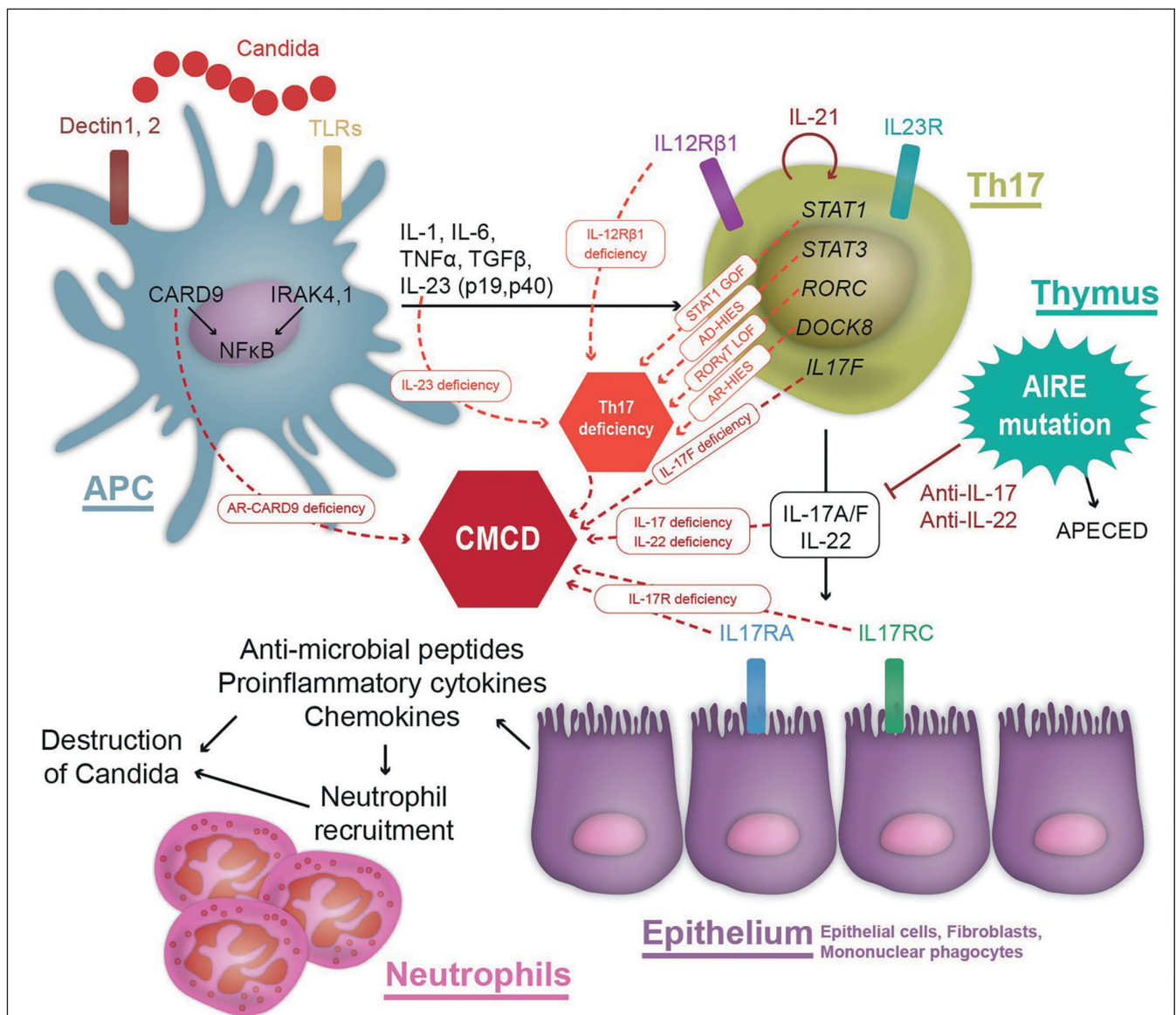
main (CCD) and DNA-binding domain (DBD) were described in a significant number of patients belonging to other kindreds (35). GOF mutations of STAT1 have been shown to be the most common etiology of CMC over the past few years (35). Heterozygous GOF mutations in STAT1 have been associated with a diverse phenotype encompassing CMC, autoimmune disease (such autoimmune hepatitis), lymphadenopathy, early-onset rosacea, and various cancers (Hodgkin Lymphoma, oesophageal cancer or squamous cell carcinoma) (36, 37).

Among CD4+ subpopulation, IL-17+ T cells impaired development resulting from CD4+ T cells were demonstrated in patients with CMC (35). These GOF mutations result in increased phosphorylation, DNA binding, transactivation, and interaction with protein inhibitor of activated STAT (PIAS), and decreased dephosphorylation of STAT1 (35). Thus, in patients with CMC, the poor development of IL-17+ T cells may be involved with increased responses to IFN-γ, IFN-α/β and IL-27, which are STAT1-dependent repressors of IL-17-producing cells (35). Lower percentages of IL-17A+, IL-17F+ and IL-22+ T cells were observed in patients with GOF STAT1 mutations, as well as a lower production of IL-6, IL-17A and IL-22 than those of healthy control subjects, while a high level of IL-4 was reported (38). Moreover, many patients with GOF STAT1 mutations that develop autoimmune disorders exhibit improved type 1 IFN cellular immune responses, which could explain the link between STAT1 hyperactivity and the development of autoimmunity (39).

STAT1 GOF mutation that is characterized as heterozygous dominant may bring about stronger cellular immune responses towards STAT1-dependent IL-17 inhibitors (IFN-α/β, IFN-γ and IL-27) (35). This is responsible for the abnormal IL-17+ T-cell development, impacting on IL-17+ T cell-mediated responses that are often antifungal in effect. The establishment of safe and effective treatments based on a precise understanding of the molecular mechanisms of this disorder is required to improve patient care (40).

Mutations in STAT3

STAT3 is a transcription factor involved in signalling for a variety of cytokines, hormones and growth factors (41). The STAT3 gene plays an important role in maturation, differentiation, and function of T and B lymphocytes (42). Sequel to STAT3 involvement in Th17 cell differentiation, the stimulation of CD4+ T cells toward the follicular helper T cell lineage may also depend on STAT3 (43). Naïve T cells differentiation towards Th17 cells lineage is a very complex process which is yet to be completely understood. STAT3 which is activated by IL-6 and IL-21, can play an essential role in the development of Th17 cells in humans (44). Moreover, STAT3 activation induces expression of RORγt which plays an important role in Th17 function (27). Two independent groups in 2007 reported that mutation in STAT3 as a major cause of sporadic HIES and autosomal dom-

inant (AD) (45). Mutations were limited in either the DBD or the Src2 homology (SH2) domains of the STAT3 gene and were later described throughout the gene in other different populations (46). Immunologic defects reported include severe increase in serum IgE levels, eosinophilia, neutrophil chemotactic defect (47), abnormal cytokine production, and abnormal antibody responses to bacteriophage ΦX174 (48). As a result, patients with AD-HIES have abnormal susceptibility to a narrow spectrum of infections, such as *Streptococcus aureus*, and *C. albicans* while this may be a likely explanation for the susceptibility of these patients to CMC. Patients with dominant negative STAT3 mutations in patients present with a decreased number of central memory CD4+ and CD8+ lymphocytes and an increased number of naïve T cells (49). Due to this memory T cell defect; these patients are predisposed to develop varicella zoster virus reactivation and prolonged Epstein-Barr virus viremia (49). Recent advances in our understanding of genetic aetiology of STAT3 mutations have established the essential role of STAT3 in Th17 cell differentiation (32). STAT3 deficient patients were assessed by Ma *et al.* for any possible potential defects in Th17 cells development (32). Examination of premature T cell produced from the total CD4+ T cells of patients showed significant decrease in IL-17+ T cells and following stimulation of these CD4+ T cells resulted in the absence of IL-17 production. Additionally, IL-17 production was impaired in both groups of HIES patients, although the impairment was more severe in patients with STAT3 mutations. Thus, defective Th17 differentiation occurs by a different mechanism in AR-HIES versus AD-HIES due to STAT3 mutations. Furthermore, the production of another Th17-derived cytokine, IL-22, involved in epithelial and mucosal immunity, was also absent from CD4+ T cells. Consistent with the vital role of STAT3 in RORγt expression, the sorted naïve CD4+ T cells from the patients failed to differentiate when stimulated with anti-CD2, anti-CD3 and anti-CD28 into Th17 T cells following the presence of IL-1β with either IL-23 or IL-6, confirming that STAT3 is a requirement for the differentiation of Th17 cells. Furthermore, cells obtained from STAT3 deficient patients and were stimulated overnight with *S. aureus* or *C. albicans* failed to generate memory CD4+IL-17+ T cells (50). NKT-17, Tc17 and Tγδ-17 are also sources of Th17 cytokines (51). However, CMC disease prevention do not require the production of Th17 cytokines or the production of insufficient quantities of Th17 cytokines in AD-HIES STAT3 mutation (8). This proposes the probability of STAT3 being involved in the production of Th17 cytokine in γδ-17 cells, Tc17 cells, and NKT-17 cells (8).

Mutations in DOCK8

DOCK8 is a member of DOCK180-related family of guanine nucleotide exchange factors (GEFs) that promotes the activity of Rho GTPases such as Rac and Cdc42 and are involved in variety

of cellular processes including cell migration, differentiation, and cell–cell interactions (52, 53). DOCK8 mediates both innate and adaptive responses to ensure sufficient immune response in that it is required for lymphocyte survival, migration, and immune synapse formation (53). Consequently, its absence results in poor pathogen control (53). Though, modern advances have pointed to an essential role of DOCK8 in regulating the signal transduction events that control transcriptional activity, cytokine production and functional polarization of immune cells (54). Biallelic mutations in the DOCK8 cause progressive combined immunodeficiency (CID) characterized by susceptibility to atopic diseases, unusual susceptibility to candidal dermatitis, recurrent respiratory infections, autoimmunity and malignancy (55-58). Recent study by Haskologlu *et al.* (55) have reported frequent clinical manifestations in subjects with DOCK8 mutations include atopic dermatitis (90%), recurrent respiratory tract infections (85%), and food allergy (70%). Failure to thrive (65%), liver problems (60%), bronchiectasis (55%), chronic diarrhea (50%), and autism spectrum disorders (15%) were strange findings. Elevated IgE level (100%) and eosinophilia (85%), low IgM level (75%), and decreased CD3+ T (50%) and CD4+ T (55%) cell count were prominent laboratory findings. The study also revealed that, stimulation with anti-CD3 and anti-CD28 in DOCK8 deficient patients leads to T cell lymphopenia accompanied by poor T cell proliferation, and exhibit decreased Th17 and memory B cells (59). A cohort study of AR-HIES patients revealed deficiencies in the differentiation of Th17 cells (60). RORγt expression, which is critical for Th17 differentiation, was significantly reduced in peripheral T cells of patients with AR-HIES. Interestingly, *in vitro* induction of RORγt expression by naïve T cells was intact. Therefore, it was suggested that initial steps of Th17 differentiation are intact in the AR-HIES patients, but subsequent steps of differentiation are impaired. It is likely that impaired Th17 differentiation and IL-17 production contributes to the susceptibility of AR-HIES patients with DOCK8 mutations to candidal dermatitis. In a more comprehensive study of functional impairment of T cell in DOCK8 deficient patients, it has been shown that the establishment of candidal infection is a result of susceptibility to viral infections. Tangye *et al.* (61) observed that DOCK8-deficient memory CD4+ T cells were partial toward a Th2 cell at the expense of Th1 and Th17 cells. Further studies into the clinical features of DOCK8-deficient CD4+ T cells concluded that the Th2 bias is likely to contribute to atopic disease, whereas defects in Th1 and Th17 cells compromise antiviral and antifungal immunity, respectively.

Mutations in AIRE

Autoimmune regulator (*AIRE)* gene is consisting of 14 exons coding for 545 amino acid protein (62). The thymic medullary epithelial cells mainly expressed the *AIRE* gene, which play a vital role in self-antigens presentation (63). In the pancreas, the *AIRE* gene is also expressed at low levels, lymph nodes, adrenal cortex, spleen and peripheral blood mononuclear cells. It is a nuclear transcriptional regulator protein involved in the ectopic expression of self-antigens in the thymus, which leads to the removal of self-reactive thymocytes and peripheral tolerance generation. The role of peripheral *AIRE* expression, which has been confirmed by mRNA analysis, is yet to be cleared. AIRE deficiency leads to the escape and extra-thymic spreading of autoreactive T cell clones that underlie the onset of autoimmune attack against several tissue-specific self-antigens (64). Worldwide, more than 100 different mutations in this gene have been reported till date, both homogeneous and heterogeneous (6, 65).

Autoimmune polyendocrine syndrome 1 (APS-1) or autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), which is an autosomal recessive disease as a result of mutations in the *AIRE* gene, characterized by CMC, adrenal insufficiency and hypoparathyroidism (65). The disease commonly begins in childhood, with the sequential onset of manifestations beginning with CMC at around five years of age, followed by hypoparathyroidism and then by adrenal insufficiency (33). Other endocrine and non-endocrine components, such as hypothyroidism, autoimmune hepatitis, type 1 diabetes, gastro-intestinal dysfunction, hypergonadotropic hypogonadism, asplenia, and various ectodermal abnormalities (nail dystrophy, intestinal keratitis, vitiligo, alopecia, and dental enamel hypoplasia) may also occur with different prevalence (66).

AIRE deficiency has been shown to enhance the differentiation of into Th1, Th17, and follicular helper T (Tfh) cells, while reducing the differentiation of Th2 cells and Tregs (67). These information propose that *AIRE* may play a role in inducing Th17 and Th1 differentiation by upregulating cytokine expression in DCs (68). Also, the autoantibodies of patients distinguish not only multiple organ-specific targets, but also most Th17 cell-associated cytokines, many type I interferons (IFNs) with neutralizing biological actions *in vitro* (69). These anti-cytokine autoantibodies are extremely disease-specific even though they have been found only in patients with thymomas, tumours of thymic epithelial cells that fail to express *AIRE*. Furthermore, autoantibodies against Th17 cell-associated cytokines associate with CMC in both syndromes (70).

The accurate diagnosis of APS-1 requires that at least two of these three major components or only one if a sibling has already been diagnosed with the disease (6). Thus, to examine if the T helper reactivity to *Candida albicans* and other stimuli was altered, PBMCs from APS-1 patients were isolated and matched with healthy controls. In APS-1 patients, the Th17 pathway was upregulated in response to *C. albicans*, whereas there is reduction in the secretion of IL-22 (71). From the sera of APS-1 patients, autoantibodies against IL-17A, IL-17F and IL-22 were detected by immunoprecipitation. Furthermore, *AIRE*-deficient mice were much more vulnerable than AIRE (+/+) mice to mucosal candidiasis and *C. albicans* induced increased responses of Th17- and Th1-cell in *AIRE*-deficient

mice. Hence, in APS-1 patients, an excessive IL-17A reactivity towards C. *albicans* was observed and *AIRE*-deficient mice (71).

Mutations in IL12Rβ1 and IL-12p40

IL-12 and IL-23 are heterodimeric cytokines composing of a subunit of p35 and p19, respectively, while both contain the same p40 subunit (72). The heterodimer, p19-p40, of IL-23 binds to IL-12Rβ1 and make use of its receptor complex comprising of IL-12Rβ1 and IL-23R on NK cells and T cells (72). IL-23 is a crucial cytokine that maintains Th17 cells expansion following differentiation from naive CD4+ cells as a result of exposure to IL-1β, IL-6, and IL-21 (44). IL-12 and IL-23 receptor b1 chain deficiency and IL-12p40 deficiency are two autosomal recessive forms of Mendelian Susceptibility to mycobacterial disease (MSMD) following moderate phenotypes with particular susceptibility to *Salmonella* infection (73). Predominantly, intracellular pathogens were found to be responsible for a wide range of infections as result of the functional impairment of IL-12Rβ1 (74). In patients with IL-12Rβ1 mutation, NK cells and T cells may not be able to respond to IL-12 or IL-23, causing the frequent development of CMC in these patients (75). Patients deficient in IL-12p40 subunit joint by IL-12 and IL-23 can also be

susceptible to CMC due to weakened maintenance of Th17 cells at mucosal surfaces (74). In a study performed by Prando *et al.* CMC was observed in 3 (6.7%) of 49 patients with autosomal recessive IL12p40 deficiency (76). One patient had invasive candidiasis and two presented with oral thrush. It was reported that 23% of patients with IL-12Rβ1 deficiency presented with clinical features of candidiasis (74). Most candidiasis episodes are mucocutaneous, oropharyngeal candidiasis, esophageal, cutaneous, or genital. In addition to mucosal or cutaneous fungal diseases, some reports of invasive candidiasis were also presented (74). The basis of immunity resulting to lower incidence of CMC in patients with IL-12p40 deficiency compared to patients with IL-12Rβ1 deficiency has not been completely understood. Both IL-12Rβ1 and IL-12p40-deficient patients with reduced IL-23 signaling, demonstrate a reduced population of Th17 cells. The reduction in the Th17 population in these patients is not as severe as in STAT3-deficient patients. This suggest that IL-23 is vital for the development of Th17 cells, maintenance, or both, but some redundancy might still exist to permit reduced development of Th17 cells. Since C. *albicans* infection is uncommon in IL-12Rβ1 and IL-12p40-deficient patients, it may be due to the bulk of these patients retaining an adequate Th17 function to prevent susceptibility to CMC. In addition, IL-12 has

Table I - *Monogenic diseases that impair Th17 development or function.*

Genetic defect	Phenotypic characteristics	References
STAT1 (GOF)	Chronic mucocutaneous candidiasis, Autoimmune hepatitis, Autoimmune hemolysis, hypothyroidism, lymphadenopathy, Early-onset rosacea, Cancers like Hodgkin's lymphoma, Oesophageal cancer or Squamous cells carcinoma, Pneumonia, *P. jirovecii* pneumonia, CMV	(36) (37)
STAT3	AD-HIES, elevated IgE, S. *aureus* abscesses, Pneumonia, Pneumatocele formation, Candidiasis	(90) (91)
DOCK8	AR-HIES, elevated IgE, Eosinophilia, Atopy, Recurrent sinopulmonary infection, Herpes virus, Candidiasis	(92) (93) (94)
AIRE	Chronic mucocutaneous candidiasis, Adrenal insufficiency, Hypoparathyroidism, Autoimmune diseases, Hypogonadism, Asplenia, Various ectodermal abnormalities, Splenomegaly, Arthralgia, Autoimmune polyendocrine syndrome 1	(33) (66) (95)
IL-17	Allergy, Psoriasis	(96, 97)
IL-17RA	Tumor cell proliferation in a JNK isoform-dependent manner, Colorectal cancer, Candidal dermatitis, *S. aureus* dermatitis	(98)
IL-22	Multiple Sclerosis	(99)
RORC	Oxazolone-induced colitis in mice Pancreatic cancer progression and lymph node invasion, Lung cancer, Breast cancer, Neuroendocrine prostate cancer	(100) (101) (101)
IL-12Rβ1 & IL-12p40 deficiency	Multiple Sclerosis, Mendelian Susceptibility to Mycobacterial Diseases	(8) (77)

been shown to play a significant role in immunity against candida (77). Last but not least, patients with IL-12p40 and IL-12Rβ1 deficiencies are evidently deficient in IL-12 responses, which account for their mycobacterial susceptibility (8).

Diagnosis of Th17 deficiency

Patients, who exclusively present with recurrent *S. aureus* and *Candida* species infection and a high IgE serum level, should be considered for the diagnosis of Th17 deficiency. Laboratory anomalies in these patients may include defective cutaneous or *in vitro* T-cell *Candida* species response. Patients with Th17 deficiency associated with STAT1, IL17RA, IL17F and CARD9 mutations will not have other defects in humoral or cellular immunodeficiencies (78, 79). Laboratory testing might reveal impaired *in vitro* lymphocyte proliferation and cytokine secretion in response to *Candida* species, while delayed-type hypersensitivity test results to *Candida* species might be normal. In patients with mutations in CARD9 and STAT1, a decrease in Th17 cell counts has been observed, although its frequency is normal in those with mutations in IL17RA and IL17F (80, 81). Immunologic abnormalities are variable in patients with DOCK8 mutations, but CID may be considered when including both cellular and humoral immune deficiencies. In a few patients with DOCK8 deficiency, TREC numbers have been found to be low (82). Based on Th17 cells surface (CD4, CCR4, and CCR6) and intracellular (IL-17 and RORC) markers, flow cytometric analysis is a valuable diagnostic tool to assess the Th17 cells frequency and function in suspect patients (83). In a study Meshaal *et al.* proposed diagnosis of DOCK8 deficiency using flow cytometry biomarkers (84). In their study, profound defects in Th17 cells and Tregs were observed in all patients with impaired STAT3 phosphorylation, indicating that DOCK8 plays a pivotal role in the STAT3 signaling pathway. These findings along with detecting diminished memory B cell numbers and defective DOCK8 expression by flow cytometry can confirm the diagnosis.

Serum levels of IL-17A and IL-22 and anti-IL-17A, anti-IL-17F, and anti-IL-22 autoantibodies can be measured by using commercially available enzyme-linked immunosorbent assay (ELISA) and chemilumiscence assays (CLIA). However, confirmation of Th17 deficiency syndromes by genetic screening of suspected patients with molecular new techniques such next generation sequencing (NGS) or evaluation of responsible genes (*STAT1, STAT3, DOCK8, AIRE, RORC, IL-17, IL-17R*, and *IL-22*) by Sanger sequencing in patients with related phenotype should not be neglected (78).

Therapeutic approach in Th17 deficiency

Treatments for monogenic patients with Th17 deficiency involve preventing and treating infections, boosting the immune system, and treating the underlying cause of the immune problem. Prolonged treatment with antifungal agents might be prerequisite, depending on the extent of *Candida* species infection. Eczem-

atous dermatitis requires rigorous topical therapy with steroids and a moisturizing cream. Topical application of calcineurin inhibitors such as Pimecrolimus and Tacrolimus may also be used for controlling eczematous lesions (78). In patients with prominent autoimmune complication such as APECED syndrome, immunomodulating drugs can also subdue clinical manifestations, but their immunosuppressive actions should be carefully monitored to avoid severe complications caused by underlying host defense abnormalities. The primary treatments for affected patients with APECED syndrome include antifungals to treat mucocutaneous candidiasis and hormone replacement for endocrinopathies (85). It was revealed that early HSCT is associated with better outcomes in some type of patients with Th17 deficiency. Successful HSCT for patients with DOCK8 mutation has been reported in several cases. HSCT has been shown to cure nearly all clinical and laboratory manifestations by reconstituting the normal function of the immune system (86, 87). In a cohort study by Haskologlu *et al.*, HSCT led to a marked improvement in atopic dermatitis and food allergies, along with decreasing infection frequency. The overall survival was 91% in HSCT-received patients (55). Recently, a study reported HSCT in cases with STAT3 mutation have had successful outcomes (88). However, in one early reported case of HSCT for STAT3 mutation, the clinical manifestations reappeared (89).

Conclusions

Th17 deficiency is an abnormality that should be considered in the diagnosis of patients who demonstrate recurrent infections and susceptibility to fungal infections. When diagnosing such patients, care should be taken in assessing the clinical symptoms, as well as the possibility of identifying the responsible genetic defect associated with the disease, and these may, eventually, result to an effective and accurate management or even treatment of the disease.

Fundings

Conflict of interests

The authors declare that they have no conflict of interests.

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IgG from atopic individuals can mediate non-atopic infant thymic and adult peripheral CD8+ TC2 skewing without influence on TC17 or TC22 cells

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Summary

The potential of IgG antibodies as allergy regulators has been discussed for decades and was brought to light that anti-allergen IgG is related to allergy inhibition in children during the first years of life and that IgG repertoire can differ between atopic and non-atopic individuals. Here, we aimed to evaluate in vitro the differential effects of purified IgG from atopic and non-atopic individuals on the production of IL-4, IL-17, and IL-22 by human intra-thymic and mature peripheral CD8+ T cells respectively termed as TC2, TC17, and TC22 cells. We additionally evaluated the IFN-γ production by CD8+ T cells. Thereupon we used infants thymic tissues from non-atopic mothers and blood samples from individuals clinically classified as non-atopic. Thymocytes or PBMCs were cultured with IgG from atopic or non-atopic individuals. As controls, we used commercial IgG (Intravenous immunoglobulin - IVIg) or mock condition. The phenotype and intracellular cytokine production were evaluated using flow cytometry. IgG from atopic individuals could increase the frequency of TC2 cells in non-atopic infant thymic and adult peripheral cell cultures compared to all control conditions. Due to the TC2 cell's potential to collaborate with pathology and severity of asthma in humans, this evidence can cooperate with the understanding of the development of an atopic state.

Introduction

Our group has been discussing the potential of IgG molecules to modulate the functional activity of thymic T cells according to the individual's immune status (1, 2). The IgG repertoire can differ between atopic and non-atopic individuals (3, 4) and its reactivity to IgE can play a pivotal role in the mechanism by which non-atopic individuals produce IgE without a response to allergen exposure (5).

It was described that IgG molecules from atopic individuals modulate *in vitro* IFN-g production by human intra-thymic TCD4 and CD8+ T cells (6) and IgG from non-atopic individuals can induce *in vitro* IFN-g and IL-10 production by human intra-thymic gdT cells (7). In a recent translational approach, it was evidenced *in vitro* that IgG from allergy-tolerant mice and allergy-tolerant humans (non-atopic) can exert similar effects upon neonatal thymocytes inhibiting the maturation of IL-17-producing gdT cells (8).

In a similar but not translational context, it was demonstrated that IgG from atopic dermatitis (AD) patients could induce the *in vitro* production of IL-17 and IL-10 by infant intrathymic TCD4, CD8+ T, and iNKT cells (9, 10).

Together, these findings indicate a broad potential of these molecules to exert modulatory functions that can directly influence the peripheral atopic immune status of mice and humans.

CD8^{+} T cells, unlike TCD4 cells, are refractory to corticosteroids (11) and have the capacity to produce IL-4 (TC2) inducing murine lung eosinophilia (12) and allergic asthma (13). In humans, TC2 cells are also related to the pathology and severity of asthma (14, 15) and increased in the airways of Chronic Obstructive Pulmonary Disease (COPD) patients (16). Indeed, human CD8^{+} T cells produce more IL-4 than TCD4 cells (17, 18), which suggests that they can play a pivotal role in the development of allergic diseases. This study aimed to evaluate *in vitro* the possible differential effects of purified IgG from atopic and non-atopic individuals on cytokine production by human intra-thymic CD8^{+} T cells that can exert some modulatory influence on allergic responses, mainly TC2 cells. We also examined whether mature peripheral CD8^{+} T cells exhibit a similar profile in response to atopic and non-atopic IgG.

Materials and methods

Thymic tissues were obtained from 13 infants who underwent corrective cardiac surgery at the Hospital do Coração (HCor), São Paulo, Brazil. The evaluated patients did not exhibit signs of immunodeficiency, genetic syndromes or allergic reactions, and patient age of fewer than 7 days was used as an inclusion criterion (patient age, mean ± standard error (SE): 3.3 ± 0.52 days). Parental allergic backgrounds were evaluated, and only children with non-atopic mothers were included in this study.

Atopic individuals were clinically classified (clinically allergic, reactive to at least two allergens in the Skin Prick Test - SPT), and blood samples were collected to confirm the atopic state by detectable titers of specific IgE to at least two allergens. Non-atopic individuals were classified in the same parameters but without any clinical allergy symptoms, not reactive to any tested allergen in the SPT and without detectable titers of specific IgE to any tested allergen. Each sample of thymic tissue or peripheral blood mononuclear cells (PBMCs) was obtained from a different donor and analyzed in three independent experiments. The ethics committees at the HCor and the School of Medicine at the University of São Paulo approved this study (CAAE: 15507613.4.0000.0060). Suspensions of thymocytes or freshly thawed separated PBMCs were washed and resuspended in RPMI 1640 medium containing 10% FCS (HyClone - Fetal Clone III, USA).

An aliquot of cell suspensions diluted in trypan blue (Sigma, USA) was evaluated to access cell viability. Thus, 1×10^6 viable cells were cultured in 96-well plate (CoStar, USA) with 100µg/mL of IgG from atopic or non-atopic individuals or in control conditions (100µg/mL of commercially used IgG or without IgG, the mock condition), all in RPMI 1640 medium containing 10% FCS.

The concentration of 100 µg/mL of IgG was previously determined as optimal to modulate the cytokine production by lymphocytes (7-9, 19) and can be considered low when compared to biological conditions (6). The culture plates were incubated for 2 days. Subsequently, 1 µg/mL Brefeldin A (Sigma, Israel) was added to each well of the culture plates, and after 24 hours, cell staining was performed to evaluate cell labeling via flow cytometry.

For extracellular staining, 0.5×10^6 cells/mL thymocytes or PBMCs were transferred to test tubes, and 1 µg of each antibody was added to the cells (except for the unlabelled tubes). Then, the samples were incubated, washed, and fixed with formaldehyde. Thymocytes or PBMCs were stained with mouse anti-human CD4 and CD8 or isotype control antibodies (BD Pharmingen, NJ, USA).

For intracellular labeling, 1 µg of each antibody was added to the cells in 100 µL of PBS containing 0.05% saponin. Thymocytes and PBMCs were stained with anti-human IL-17A, IL-22, and IL-4 or isotype control conjugated with fluorochromes (BD Pharmingen). Detailed method describing SPT, serum anti-allergen IgE determination, collection of blood samples, separation of human PBMC and thymocytes suspensions, determination of human total IgG subclasses, IgG purification, and additional cell culture and flow cytometry methods aspects can be accessed on the **online supplements** (**Methods** and **References**).

Results

First, we evaluated atopic and non-atopic IgG subclasses and could observe that they can be found at similar levels between purified IgG samples (**online supplements figure 1S**). Next, Thymocytes or PBMCs were cultured in the presence of purified IgG for three days, and, after this period, we found that the culture conditions used in this study did not influence the frequency and viability of thymic nor peripheral CD8^{+} T cells (**online supplements figure 2S**).

After evaluating cell frequency and viability, we analyzed cytokine production in CD8^{+} T cells. We found that treatment with IgG from atopic individuals induced elevated production of IL-4 in infant thymic and adult peripheral CD8^{+} T cells compared to non-treatment and treatment with IVIg or IgG from non-atopic individuals (**figure 1 A, B**). IL-17 and IL-22 production on infant thymic and adult peripheral CD8^{+} T cells did not appear to be influenced by any of the evaluated IgG. We also assessed IFN-γ production, and we could observe that IgG from non-atopic individuals induced an augmented production of IFN-γ in infant thymic CD8^{+} T cells when compared to non-treatment and treatment with IVIg or IgG from atopic individuals. No effect could be observed on IFN-γ production by adult peripheral CD8^{+} T cells.

Discussion

Although CD8^{+} T cells are essential compounds of adaptive immunity contributing to clearance of intracellular pathogens and long-term protection, there is growing evidence suggesting that

Figure 1 - *Effect of purified IgG on non-atopic infant intrathymic and non-atopic adult peripheral CD8+ T cells.*
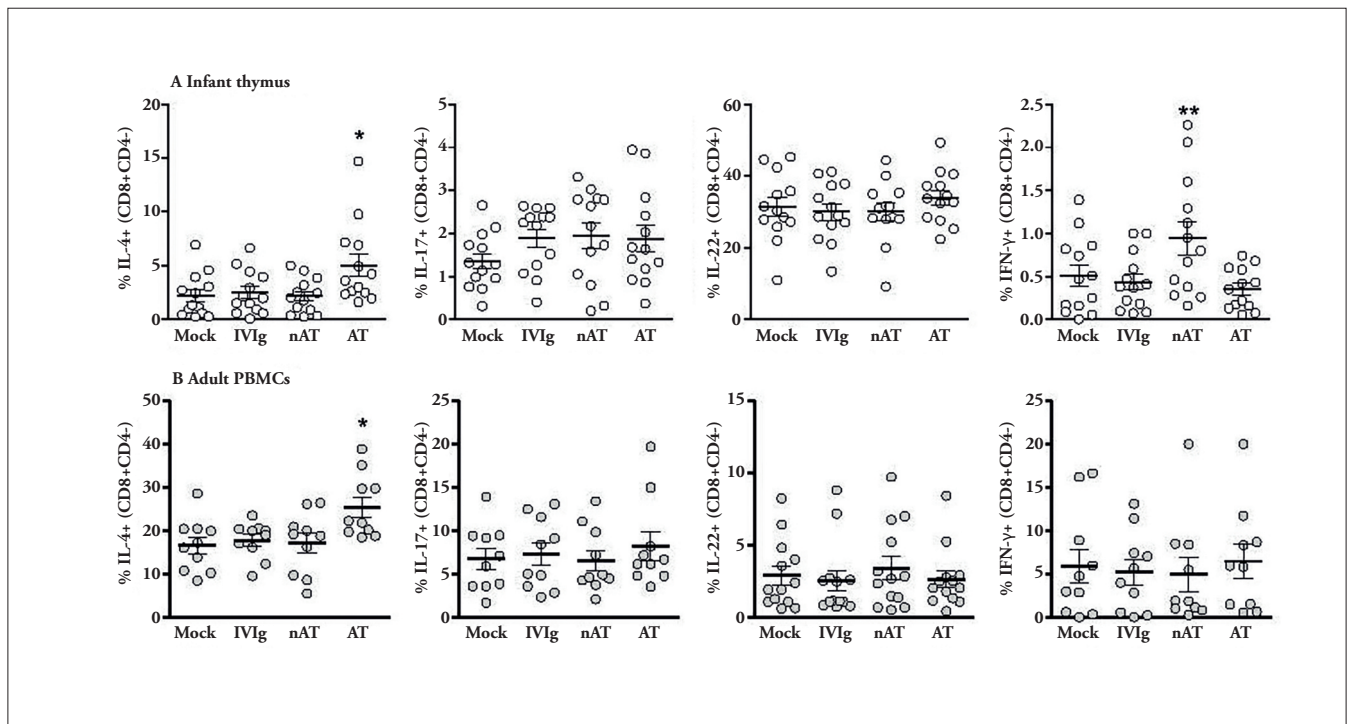
Thymocytes from infants less than 7 days old (n = 13) or PBMCs from adults (n = 10) were cultured for 3 days in RPMI medium supplemented with FCS. Those cells were cultured in the absence of IgG (mock), in the presence of 100 μg/mL of commercial IgG (IVIg), 100 μg/mL of IgG from non-atopic individuals (nAT) or 100 μg/mL of IgG from atopic individuals (AT). The frequency of cultured thymic (CD8+CD4-) CD8+ T cells (A) and peripheral (CD8+) CD8+ T cells (B) that produces IL-4, IL-17, IL-22 and IFN-γ were evaluated. The results demonstrate individual values, and the lines represent the means with standard error. *P ≤ 0.05 compared with the mock, IVIg and nAT conditions. **P ≤ 0.05 compared with the mock, IVIg and AT conditions.

IL-4-producing CD8+ T (TC2) cells can exert a pivotal role in the regulation of allergy development (20).

The possibility of IgG as allergy regulators has been discussed for decades, and anti-allergen IgG shown to be related to allergy inhibition in children during the first years of life (21-23). Here we demonstrate that IgG from atopic individuals can induce the thymic maturation of TC2 cells *in vitro*. In humans, TC2 cells are related to the pathology and severity of asthma (14, 15) and increased in the airways of Chronic Obstructive Pulmonary Disease (COPD) patients (16).

Human CD8+ T cells were already described significant producers of IL-4 when compared to TCD4 cells (17, 18), although this is not consensual in the literature, our observations corroborate with the emerging evidence suggesting a pivotal role of TC2 cells in allergic diseases as cited above.

To elucidate if the observed results could be better related to allergy development, we also observed if IgG from atopic individuals could modulate the maturation of IL-17-producing CD8+ T cells (TC17) that are related to the initiation and maintenance of autoimmunity diseases (24-26). Our findings indicated that IgG from atopic individuals could not influence the maturation of autoimmune-related TC17 cells.

We also evaluated if IgG from atopic individuals could modulate the maturation of IL-22-producing CD8+ T cells (TC22), a CD8+ T subset related to the development of a non-related atopic disease characterized by chronic skin inflammation, the Atopic Dermatitis (27). Since we did not observe any influence in the frequency of TC22, these results corroborate with the hypothesis that IgG from atopic individuals can mediate a mechanism restricted to allergy development.

Due to the significant importance of IFN-γ in allergy development, we also evaluated the production of this cytokine. Interestingly, IgG from non-Atopic individuals could induce augmented levels of this cytokine in infant thymic but not adult peripheral CD8+ T cells. A similar effect mediated by non-Atopic IgG on thymic CD8+ T cells was already described in the literature and can corroborate with the development of a non-Atopic as held by IgG donors (6). The IgG-mediated IFN-γ modulation on thymic CD8+ T cells was also described using IgG from HIV infected individuals (28).

Taken together, the effects of purified atopic and non-atopic IgG, suggests that IgG from atopic individuals induces IL-4-producing thymic CD8^{+} T cells. In contrast, IgG from non-atopic individuals induces IFN-γ-producing CD8^{+} T cells. These observations corroborate with the higher frequency of IL-4-producing, and the lower frequency of IFN-γ-producing CD8^{+} T cells demonstrated in patients with allergic asthma when compared to healthy controls (29).

In the present paper, we did not demonstrate the effect of IgG from atopic and non-atopic individuals on the modulation of TCD4 cells. Still, it was shown in a very similar *in vitro* approach that IgG from atopic individuals can also modulate thymic TCD4 cells inhibiting IFN-γ and inducing IL-17 production (6).

If transposed to *in vivo* conditions, our findings can collaborate with the elucidation of allergy development in atopic individuals. This mechanism can involve the event of an atopic-prone IgG repertoire that can, since the neonatal period, favor the maturation of TC2 cells that reaches the peripheral tissues and collaborates with allergy development.

In this context, we can also suggest a role for maternal immunity since maternal IgG modulates children immune status by the passive transference during the gestational period (3, 21, 30-34). As theoretically discussed in 2017 with the "MatIgG primary modulation theory" (35), the passive transference of maternal IgG can modulate offspring immunity when maternal IgG reaches offspring primary organs modulating the immunity development according to maternal atopic state. This hypothesis has been confirmed with pieces of evidence obtained in murine (36, 37) and translational experimental approaches (38, 39).

Conclusions

Our observations are unprecedented in the literature and corroborate with studies that had demonstrated IgG as a molecule that modulates the thymic maturation of lymphocytes favoring the development of allergy. Furthermore, it yields novel but still exploratory observation, which can collaborate with the future elucidation of atopic state development in humans.

Acknowledgments

Conflict of interests

The authors declare that they have no conflict of interests.

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Methods

SPT, serum anti-allergen IgE determination and collection of blood samples

The SPTs were performed in accordance with European standards (1) with an adapted panel of allergens that included the profile of Brazilian allergens (*i.e., Dermatophagoide farinae, Dermatophagoides Pteronyssimus, Blomia tropicalis, Gallus gallus domesticus, Litopenaeus vannamei, Farfanepenaeus subtilis, Farfanepenaeus paulensis, Sus scrofa domesticus, Bertholletia excels, Arachis hypogaea, Aspergillus fulmigatus, Penicillium notatum, Alternaria Alternata, Cladosporium herbarum, Blatella germânica, Periplaneta Americana, Avena nativa, Hordeum vulgare, Tricicum aestivum, Gallus gallus domesticus, Solenopsisi invicta, Culex pipiens, Canis familiaris Felis domesticus* and *Bos Taurus*) as described previously by our group (2). We excluded patients who used antihistamines, glucocorticosteroids or certain other systemic drugs that can influence the SPT results within 15 days before the test. We also excluded volunteers with severe eczema or dermographism.

Serum-specific IgE antibodies have been measured with a multiplex immunoblot assay (EUROLINE Inhalation 2 - EUROIMMUN AG, Lubek, Germany). The tested extracts were: *Dermatophagoide farinae, Dermatophagoides Pteronyssimus, Aspergillus fulmigatus, Penicillium notatum, Alternaria Alternata, Cladosporium herbarum,* cat, dog, horse, guinea pig, rabbit, hamster, timothy grass, cultivated rye, alder, birch, hazel, mugwort, english plantain and cage bird mix (budgerigar, chicken, canary bird and goose).

IgE determination was performed according to the specifications of the EUROLINE Inhalation 2 Kit (EUROIMMUN, Lubek, Germany) to obtain semi-quantitative results.

After confirmation of the individual atopic state, blood samples were obtained from each individual via venepuncture.

Separation of human PBMC and thymocyte suspensions

PBMC and thymocyte separations were performed using Ficoll-Paque Plus (GE Healthcare, Sweden) after centrifugation, as previously described by our group (2). Cell viability was assessed by flow cytometry.

Determination of human total IgG subclasses

Total IgG subclasses were measured according to the specifications of the BINDARID Radial Immunodiffusion Kit (RID - Binding Site, UK). Briefly, 5 mL of each purified IgG pool was applied to a subclass-specific RID plate and incubated at room temperature for 72 hours. Ring diameters were measured, and the concentrations were determined using a reference table provided in the kit. Calibrators supplied with the kit were used as controls.

IgG purification

IgG was purified from pooled serum samples according to the specifications of the Melon Gel IgG Spin Purification Kit (Thermo, USA) and as previously described by our group (3, 4). All samples were collected, sterilized, and stored at - 80°C for subsequent use in cell culture experiments. The IgG concentration was determined using Coomassie Protein Assay Reagent (Pierce, USA) according to the manufacturer's instructions. The purity of IgG, evaluated by SDS-PAGE, was above 95%. IgA, IgE, and IgM were undetectable on samples after purification.

Additional Cell culture methods aspects

For cell viability analysis, extracellular staining was performed as described above, and cells were incubated with Live/Dead (PE-Texas red) fluorescent reagent (ThermoFisher, USA). All extracellular and intracellular analyses were performed using viable cells.

Additional flow cytometry methods aspects

To standardize intracellular cytokine detection, we applied stimulation with PMA and ionomycin for 6 hours as a positive control (5). As a negative control, we used *ex vivo*-stained cells. Positive controls induced 4- to 6-fold percentages of all evaluated cytokines on TCD8 cells with similar mean fluorescence intensity (MFI) levels. In contrast, in the negative controls, the rates of all evaluated cytokines on TCD8 cells were 0.5% or smaller. Compensation for the instrument was performed using microbeads adsorbed with anti-mouse antibodies (CompBeads, BD Biosciences) and their conjugated antibodies. Acquisition of 300,000 events per sample was conducted in the lymphocyte quadrant (as determined by ratio size/granularity) on an LSR-Fortessa cytometer (BD Biosciences, USA), and analysis was performed using FlowJo software 10.1 (Tree Star).

All antibodies were titrated to determine the optimal concentration for specific staining. Cell gating was based on the isotype control values and the FMO setting.

Statistical analysis

Comparisons among the evaluated groups were performed ANOVA method to compare all pairs of columns. Statistical significance was defined as a p-value < 0.05 determined using GraphPad Prism software (CA, USA).

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Figure 1S *- IgG subclasses of purified IgG. IgG from atopic or non-atopic adults was pooled, and radial Immunodiffusion evaluated IgG subclasses. Data are presented as means ± SEM.*
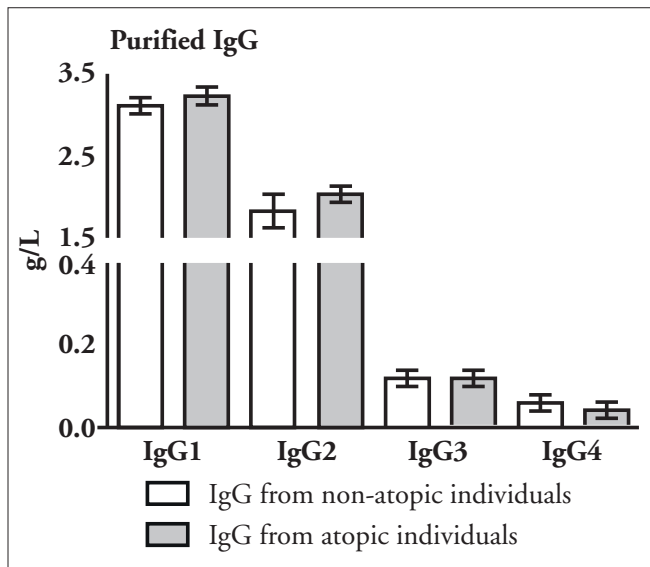
Figure 2S *- Effect of purified IgG on non-atopic infant intrathymic and non-atopic adult peripheral TCD8 cells. Thymocytes from infants less than 7 days old (n = 13) or PBMCs from adults (n = 10) were cultured for 3 days in RPMI medium supplemented with FCS. Those cells were cultured in the absence of IgG (mock), in the presence of 100 μg/mL of commercial IgG (IVIg), 100 μg/mL of IgG from non-atopic individuals (nAT) or 100 μg/mL of IgG from atopic individuals (AT). The frequency and viability of cultured TCD8 cells were evaluated on thymocytes (A) and PBMCs (B). The results demonstrate individual values, and the lines represent the means with standard error.*
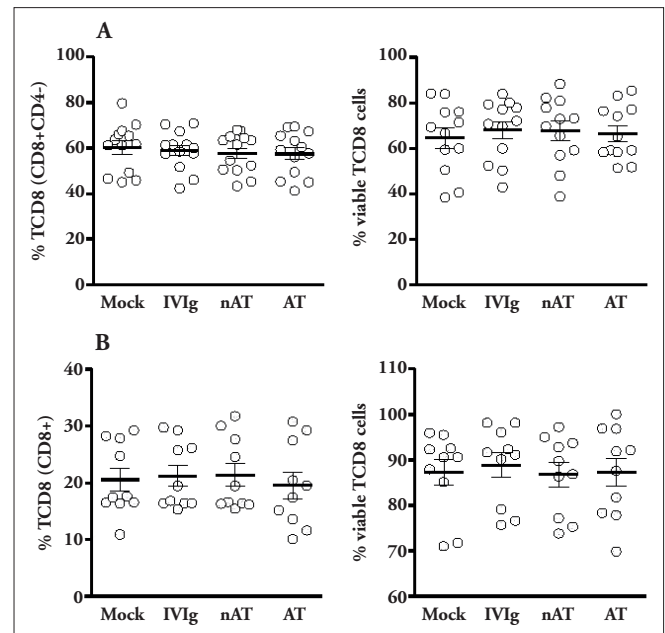
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Evaluation of two commercial peach extracts for skin prick testing in the diagnosis of hypersensitivity to lipid transfer protein. A multicenter study

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Summary
The clinical usefulness of two commercial peach extracts for SPT (by Lofarma SpA and ALK-Abellò, respectively) was compared in a multicenter study carried out in Italy. Peach allergic patients were tested with the two extracts in parallel and underwent the detection of IgE specific for all three peach allergens currently available (Pru p1, Pru p3, and Pru p4, respectively). The two extracts were almost identical in terms of sensitivity and specificity, being able to detect virtually all patients sensitized to stable peach allergens (lipid transfer protein (LTP) and, presumably, peamaclein) but scoring negative in patients exclusively sensitive to labile allergens (either PR-10 and/or profilin). Thus, the two extracts represent an excellent tool to carry out a preliminary component-resolved diagnosis of peach allergy at the first patient visit.

Introduction

In the clinical routine practice, the diagnosis of allergic diseases is based on a) convincing clinical history, b) demonstration of hypersensitivity to one or more allergen sources, and c) provocation tests (in specific cases only). The detection of IgE-mediated hypersensitivity is traditionally (and still in most cases) accomplished by skin prick tests (SPT) with commercial extracts of different allergen sources. The in-vitro detection of IgE specific for either allergen sources or single allergen components is generally reserved to cases in which skin testing is not feasible (*e.g.*, during antihistamine treatment) or in the presence of skin reactivity to a large number of different sources due to sensitization to cross-reacting pan-allergens. The EU decree 2001/83/EG establishes that solutions for skin tests are legally assimilable to drugs and must be licensed by individual national regulatory agencies based on strict quality control. The elevated costs of regulatory requirements are leading to the gradual withdrawal of the less commonly employed preparations by the producing companies and, obviously, the loss of diagnostic *in vivo* extracts has involved mainly food allergen extracts. The lack of commercial SPT food extracts can be replaced by SPT with fresh material, which is unfortunately often not feasible, or by *in vitro* tests with an undeniably significant increase in costs for both the patients and the NHS, and of more time wasted by patients and doctors. Thus, the availability of newly licensed food extracts for SPT is welcome.

Recently, the Italian regulatory agency, AIFA (Agenzia Italiana del Farmaco) admitted to the licensing process a commercial peach extract for SPT produced by Lofarma, Milan, Italy. The availability of commercial peach extracts for SPT in a Mediterranean country like Italy is of the utmost importance, as they most likely lack labile allergens (*i.e.*, Pru p1, the PR-10 allergen homologous to the major birch pollen allergen, Bet v1, and Pru p4, the peach profilin) which are lost during the production procedures while retaining stable allergens like Pru p3 (the nonspecific Lipid Transfer Protein, LTP) and Pru p7 (the gibberellin-regulated protein, also known as peamaclein) (1-3). Therefore, SPT with these extracts represents an essential means to carry out a prompt, first-level component-resolved diagnosis at the bedside, getting immediately very important information from a clinical and prognostic point of view (1, 4). Notably, LTP is by far the most frequent primary food allergy in Italy (5), and the main cause of food-induced anaphylactic reactions (6). So far, clinical studies dealing with food allergy induced by *Rosaceae* or with allergy to LTP have been carried out using a commercial peach extract enriched with Pru p3 (declaring a concentration of 30 mg/ml Pru p3) by ALK-Abellò, Madrid, Spain (7-10). In the present multicenter study, we analyzed another commercial peach extract produced by Lofarma, Milan, Italy comparing its clinical usefulness with that of the mentioned ALK-Abellò extract in a large population of peach-allergic patients sensitized to different peach allergens.

Methods

Commercial peach peel SPT extract

The commercial peach (pulp + peel) extract for SPT was prepared by Lofarma S.p.A. laboratories, Milan Italy. The final concentration of Pru p3 in this extract is adjusted to 50 μg/ml before commercialization.

The peach extract was distributed to the allergy centers scattered throughout the country who participated in the study.

Patients and skin tests

Patients with a convincing clinical history of peach allergy (*i.e.*, clear-cut oral allergy syndrome, urticaria/angioedema, or anaphylaxis following by less than 2 hours the ingestion of peach) were enrolled in the study. After signing an informed consent, patients underwent SPT with both Lofarma and ALK-Abellò peach extracts. Skin prick tests were carried out following established methods using disposable commercial 1 mm tip lancets (the single centers were left free to use their usual commercial lancets). The two commercial peach extracts were tested in parallel. Histamine 10 mg/ml and saline were used as positive and negative controls, respectively. Readings were taken at 15 min, and the mean diameter of the wheal was measured. Wheals exceeding 3 mm were considered positive.

In vitro tests

IgE to Pru p1 (or Bet v1), Pru p3, and Pru p4 (or Phl p12) were measured by ImmunoCAP. Levels exceeding 0.1 kU/L were considered positive. Patients scoring positive for Pru p1/Bet v1 and/or Pru p4/Phl p12 in the absence of detectable reactivity to Pru p3 were considered as possibly sensitized uniquely to labile peach allergens.

Results

Two-hundred forty-four peach-allergic patients (M/F 155/89; mean age 30.7 years, range 5-68 years) were finally enrolled. Of these, 220 scored positive on SPT with both (n = 216) or one (n = 4) peach extract showing an almost perfect agreement (99.5%; p < 0.0001) between the two tests. In the four patients showing a discrepancy between the two SPT extracts, Lofarma extract scored negative in 3 cases and ALK-Abellò extract in 1 case. These 4 patients showed Pru p3 IgE levels ranging from 0.45 to 3.2 kU/L. Although this did not cause appreciable differences in sensitivity between the two study extracts, several centers reported that the size of the wheals produced by the ALK-Abellò extract frequently slightly exceeded those induced by the Lofarma extract.

Patients scoring positive for one or both peach SPT extracts showed IgE to Pru p3 in 198/202 (98%) cases; 4 patients did not show any IgE reactivity to PR-10, profilin, or Pru p3 on ImmunoCAP analysis despite being strongly sensitized to peach (both extracts positive

on SPT) and having clinical history of systemic reactions. Therefore, they were classified as being sensitized to a stable allergen other than the lipid transfer protein, possibly peamaclein or another yet not identified peach allergen (10). IgE to Pru p3 was not measured in 18 patients (all scoring positive on SPT with both extracts).

Twenty-four patients were sensitized exclusively to labile allergens (*i.e.*, they showed IgE reactivity to either PR-10 and/or Profilin in the absence of IgE reactivity to Pru p3, the peach LTP). All of them scored negative on skin tests with both peach extracts. The results of the study are summarized in **table I**.

Table I - *Skin and serological tests carried out in 244 peach-allergic patients.*

ImmunoCAP	Lofarma SPT+	ALK SPT+
Pru p3+ (n = 198)	195	197
Pru p1 or 4+/Pru p- (n = 24)	0	0
Pru p1, 3, 4- (n = 4)	4	4
Not tested (n = 18)	18	18

Discussion

Clinical allergists strongly need instruments able to identify already at the first visit whether the patient presenting for a consultancy has an allergy or not. The license regulations decreed by the EU and adopted by the National regulatory agencies pose a serious risk to make this task extremely difficult or even impossible with a consequent exponential increase of costs for the patients and the community and of time expenditures by both doctors and patients. Therefore, the availability of diagnostic instruments for *in vivo* diagnosis of food allergy is welcome. In the present study, we compared the clinical usefulness and performance of two commercial peach extracts, one by Lofarma SpA and the other by ALK-Abellò in a large group of peach-allergic patients. The two tests showed a nearly identical efficiency: both scored negative in patients sensitized uniquely to labile peach allergens and positive in most patients sensitized to stable peach allergens. This feature of the two preparations allows to carry out a component-resolved diagnosis of peach allergy at the first clinical visit, identifying patients who are at a greater risk of systemic reactions. Interestingly, both extracts were able to identify four patients sensitized to stable peach allergens other than LTP.

Conclusions

The study confirmed that peach labile allergens are lost during the extraction procedures and that SPT with fresh material are needed to detect patients sensitized to such allergens in the clinical practice. The main limitation of this study is the lack of a correlation analysis between the level of IgE specific for LTP and the size (either area or mean diameter) of the wheal produced by the two extracts on SPT. However, in view of the high variability of skin responses between patients showing equal levels of specific IgE, it seems unlikely that such analysis would have added further useful information to the current findings. Therefore, it is possible to conclude that both peach extracts studied are clinically useful to detect/exclude sensitization to stable peach allergens.

Conflict of interests

The authors declare that they have no conflict of interests.

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Delayed corticosteroid hypersensitivity: a clinical management proposal

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Summary

Background. *Different clinical pictures are related to corticosteroids (CS) non immediate hypersensitivity and the frequency of these reactions can be underestimated. The classification of CS in 3 groups and the identification of two patient's profiles has been proposed by Baeck to help clinicians in the management of these cases.* **Methods.** *Data of 14 patients with clinical history of delayed reactions to various CS and positive skin test and/or oral challenge are retrospectively analyzed.* **Results.** *Three different patterns of patients are identified evaluating history, clinical picture and tests results. The first one (6 pts, 43%) is characterized by cutaneous and/or mucosal reaction due to inhaled Budesonide and patch test positive only to topical molecules belonging to the group 1 of CS. The second pattern (4 pts) has clinical history of local and systemic skin reactions to the topic and parenteral administration of the same or other steroid drugs. Patients belonging to the third pattern (4 pts) have a history of systemic reactions to general administration of CS without previous contact reaction. Patterns 2 and 3 show a wide sensitization to molecules belonging to the 3 groups of CS. All the patients show patch test positive to Budesonide.* **Conclusions.** *Although the lack of standardization, the allergy workup proves useful to differentiate patients sensitized to one or few molecules from polysensitized and to identify the culprit drugs. Intradermal and challenge test are necessary to complete the diagnostic workup. The results suggest the possibility of a different management of patients. Patients of pattern one can be only patch tested with a limited series of CS belonging to the 3 groups. They don't need an extensive exclusion of steroids use. The pattern 2 and 3 must be submitted instead to a complete allergological individual evaluation to identify alternative tolerated drugs, because of the risk of systemic reactions. The Baeck's classification shows limited usefulness in these cases.*

Introduction

Although it seems a paradox, several cases of allergy to corticosteroids (CS) are described and both the IgE-mediated and the cellular type IV mechanisms are involved, causing immediate and delayed hypersensitivity reactions (1, 2) respectively. Steroids hypersensitivity is rare despite their wide use, in particular immediate reactions for which the prevalence is 0.1-0.3% (3); delayed reactions are more frequent with prevalence being 0.1-5% (4). However, the reactions could be under-diagnosed, since the clinical presentation can be sometimes confused with the disease requiring the steroid treatment. Delayed reactions can be due to topical application (allergic contact dermatitis, ACD) or can be the consequence of systemic administration of steroid to a patient previously sensitized. This is known as systemic contact dermatitis (SCD) and it can affect the 5% of cases with CS contact allergy (4).

Every CS molecule can cause sensitization and both topic and systemic administration can elicit symptoms. Three generations of steroids, in addition to cortisol (hydrocortisone) and cortisone (11-deidrocortisol) are available. The first classification (Coopman 1989) in 4 groups in function of their structure and contact-allergenic properties, has been modified by Baeck in 2011 into three groups (5) (**table I**). Steroids belonging to

group 1 are more prone to cause hypersensitivity due to the absence of C_{16}-methyl substitution and non-halogenation leading to an easier link to proteins and consequent aptenization. In the same study, Baeck identified two profile of patients: profile 1, patients who react to steroids from one group only, for whom the cross-reaction seems due to electrostatic fields and profile 2, patients who react to two or three groups because of the immunological recognition of the whole skeleton structure of steroid molecule (5). How this classification is useful in the clinical practice for predicting reactions is not clear. In fact, the cross-reactivity among steroids is important for delayed reactions, while less is known for immediate ones. Moreover, due to the lacking guidelines, the standardisation of diagnostic tests is still a problem.

The study retrospectively describes 14 cases of different delayed reactions, with the aim to better understand how to manage these patients in the clinical practice.

Secondary purpose is to compare the clinical pattern of these patients with the two patients profiles proposed by Baeck (5).

Materials and methods

Data of 14 patients with delayed steroid reactions confirmed by positive skin test and/or oral challenge were retrospectively collected. The mean age of the group was 60 years, nine were female. Written informed consent was obtained from each patient. Almost all patients (13 out of 14) had been patch tested with the CS molecules listed in **table II**, representative of the three groups of steroids according to the Baeck classification (5), with the addition of other drugs based on the individual clinical history. Standardized extracts for patch test were available only for a few molecules (Budesonide, Hydrocortisone 17 and 21, Desametasone), while for the others the commercial drugs were used according to the European guidelines criteria (6). The subsequent diagnostic process was personalized on the history and the clinical feature of each patient. Ten patients underwent to skin prick test (SPT), intradermal test (ID) with immediate and late reading (until 96 hours) and oral challenge (OC) for one or more drugs (7).

Table I - *Corticosteroid classification. Adapted from Baeck (5).*

Group 1	Group 2	Group 3
Budesonide	Triamcinolone	Alclometasone dipropionate
Cloprednol	Amcinonide	Beclomethasone dipropionate
Cortisone acetate	Desonide	Betamethasone
Dichlorisone acetate	Fluchloronide	Betamethasone 17-valerate
Difluprednate	Flumoxonide	Betamethasone dipropionate
Fludrocortisone acetate	Flunisolide	Betamethasone sodium phosphate
Fluorometholone	Fluocinolone	Clobetasol propionate
Fluprednisolone acetate	Fluocinonide	Clobetasone butyrate
Hydrocortisone	Halcinonide	Desoximetasone
Hydrocortisone aceponate	Triamcinolone acetonide	Dexamethasone
Hydrocortisone acetate	Triamcinolone benetonide	Dexamethasone acetate
Hydrocortisone 17-butyrate	Triamcinolone diacetate	Dexamethasone sodium phosphate
Hydrocortisone 21-butyrate	Triamcinolone hexacetonide	Diflucortolone valerate
Hydrocortisone hemisuccinate		Diflorasone diacetate
Isofluprednone acetate		Flumethasone pivalate
Mazipredone		Fluocortin butyl
Medrysone		Fluocortolone
Methylprednisolone acetate		Fluocortolone capraylate
Methylprednisolone aceponate		Fluocortolone pivalate
Methylprednisolone hemisuccinate		Fluocortolone acetate
Prednicarbate		Halometasone
Prednisolone		Meprednisone
Prednisolone caproate		Fluticasone propionate
Prednisolone pivalate		Mometasone furoate
Prednisolone sodium metasulfobenzoate		
Prednisolone succinate		
Prednisone		
Tixocortol pivalate		

Table II - *Panel of steroids used for patch test.*

Group 1	Group 2	Group 3
Budesonide	Triamcinolone acetonide	Betamethasone dipropionate
Hydrocortisone	Flunisolide	Betamethasone sodium-phosphate
Hydrocortisone 17 butyrate		Desoxymethasone
Hydrocortisone 21 butyrate		
Methylprednisolone acetate		
Methylprednisolone hemisuccinate		
Prednisolone		

Table III - *Characteristics of patients belonging to the Pattern 1.*

N°	Age	Sex	Culprit drug	Clinical presentation	Positive patch test	Intradermal test	Oral challenge
1	55	F	Budesonide aerosol	Face and neck erithema and oedema	**Budesonide Hydrocortisone-21-acetate**	Hydrocortisone succinate negative	Prednisone negative
2	36	F	Budesonide aerosol	Face and neck erithema Lips oedema	**Budesonide**	Betamethasone phosphate negative	Deflazacort negative
3	23	F	Budesonide aerosol	Oral and face oedema	**Budesonide Hydrocortisone- 17 butyrate**	N. D.	N. D.
4	42	F	Budesonide aerosol	Lips oedema Stomatitis	**Budesonide Hydrocortisone- 17 butyrate**	N. D.	N. D.
5	49	M	Nasal Budesonide	Face oedema and mucosal oedema	**Budesonide**	Betamethasone phosphate negative	Betamethasone phosphate negative
6	58	F	Budesonide aerosol	Lips oedema	**Budesonide**	N. D.	N. D.

The culprit drug is underline when positive; N.D.: not done.

Results

According to clinical history, culprit drugs and test results, the patients were divided into three patterns.

Pattern 1 (see table III)

Six patients with local reaction of various degree of severity appeared after Budesonide bronchial inhalation (5 cases) or nasal spray (1 case).
Patch test confirmed the causal relationship with Budesonide. The cross-reactivity was limited to a few molecules belonging to the same group of CS such as Hydrocortisone 17-butyrate and Hydrocortisone 21-acetate, both used locally. Prick test, ID and oral challenge test performed with a set of different molecules resulted negative for three patients.

Pattern 2 (see table IV)

Four patients with a history of ACD due to steroids other than Budesonide, for topic skin use. Two of them presented systemic skin reactions after parenteral administration of other steroids. Patch test showed a wide polisensitization in three patients. Patient number 7 underwent intradermal test only (negative for the culprit but positive for a not suspected molecule) while oral challenge protracted for two days was necessary to confirm the history of systemic dermatitis elicited by oral desamethasone.

Pattern 3 (see table V)

Four patients with systemic delayed urticaria or exanthema after administration of different systemic drugs, without any previous local reaction to topical products.

Table IV - *Characteristics of patients belonging to the pattern 2.*

N°	Age	Sex	Culprit drug and administration route	Clinical presentation	Positive patch test	Intradermal test	Oral challenge
7	77	M	**Topical** not known **Desamethasone** oral	Allergic Contact dermatitis Systemic dermatitis	N. D.	**M- prednisolone positive** Desamethasone negative	**Desamethasone positive**
8	75	F	**Betamethasone valerate** topical **Betamethasone Na- phosphate** parenteral	Allergic Contact dermatitis Diffuse Erithema	**Budesonide** **Beclomethasone** **Fluocinolone a.** **Flunisolide** **Triamcinolone a.**	**Betamethasone phosphate positive** Prednisone negative M- prednisolone negative	Prednisone negative
9	62	F	**Topical** drugs not known	Allergic contact dermatitis	**Budesonide** **Betamethasone valerate** **Betamethasone dipropionate** **Deflazacort** **Desamethasone** **Diflucortone** **Hydrocortisone** **M-prednisolone** **Triamcinolone**	N. E.	N. E.
10	62	F	**Betamethasone valerate** topical	Allergic contact dermatitis	**Betamethasone valerate dipropionate Na phosphate** **Beclomethasone** **Budesonide** **Desamethasone** **Diflucortolone** **Hydrocortisone 17 butyrate** **Prednisolone** **Triamcinolone**	Betamethasone Na Phosphate negative M- prednisolone negative	Prednisone negative

The culprit drug is underline when positive; N.D.: not done.

They showed wide sensitization to the patch test panel, comprehensive of the suspected drug, while intradermal test was negative to the culprit drug in three cases and challenge test gave doubtful results.

Discussion

Drug allergy reactions to steroids are not so rare, mainly the delayed ones. Allergic contact dermatitis is the most frequent clinical picture: it has to be considered in patients using cutaneous topical steroids with worsening of dermatitis and also in patients using inhaled steroid and developing mucositis, cutaneous eczema or angioedema involving nasal or oral mucosa, lips, face and neck. Moreover, a systemic cutaneous reaction can be caused by systemic administration, even after a single dose of steroid drug, used for example for intra-articular injection (1, 2, 4, 8).

The cases analyzed in this study confirm that delayed hypersensitivity to CS can be caused by different patterns of sensitization and can occur with different clinical picture. One of the most frequent (pattern 1: 43%) is the muco-cutaneous reaction to inhaled products, with Budesonide resulting the sensitizer molecule and a cross-reactivity limited to a few similar topical steroids, belonging to the same group. The clinical consequence is that these patients don't seem to be prone to develop systemic reactions and usually they should not limit the use of steroids. In the second group of patients a previous local reaction to a cutaneous product can lead

Table V - *Characteristics of patients of pattern 3.*

N°	Age	Sex	Culprit drug and administration route	Clinical presentation	Positive patch test	Intradermal test	Oral challenge
11	68	M	**Triamcinolone intraarticular**	Delayed urticaria	**<u>Triamcinolone</u> Desamethasone Diflucortolone Betamethasone Na phosphate Betamethasone valerate Betamethasone dipropionate Budesonide Hydrocortisone 17 butyrate M-prednisolone a M- prednisolone s.**	**M- prednisolone positive** Prednisone negative Triamcinolone negative Betametasone Na phosphate negative	**Prednisone negative**
12	81	M	**Betamethasone Na phosphate** intraarticular	Delayed urticaria	**<u>Betamethasone</u> Budesonide Beclomethasone Desamethasone Diflucortone Fluocinolone Flunisolide Hydrocortisone Methylprednisone Triamcinolone**	**<u>Betametasone Na phosphate</u> positive** M- prednisolone hemisucinate negative	Deflazacort doubtful
13	71	F	**M-prednisolone** parenteral **Deflazacort** oral	Diffuse exanthema	**Budesonide Hydrocortisone 17 butyrate <u>Deflazacort</u>**	M- prednisolone negative Betametasone Na phosphate negative	Betametasone Na phosphate negative
14	76	M	**Betamethasone Na-Ph** parenteral **Desamethasone** oral **Beclomethasone** rectal	Urticaria Immediate rash Urticaria	**Budesonide <u>Beclomethasone</u> M- prednisolone**	M- prednisolone negative Betametasone Na phosphate negative	Prednisone doubtful

The culprit drug is underline when positive; N.D.: not done.

to systemic symptoms when the drug is administered by systemic route; this could be triggered even by drugs different from the sensitizer. These patients show sensitization to a wide range of molecules belonging to different chemical groups of CS.

The third pattern is characterized by a history of delayed urticaria or exanthema after systemic administration of various steroid drugs by different route (oral, intra-articular, intramuscular, rectal). No previous topical reactions are reported and patients show sensitization to many molecules over the culprit. All the patients are sensitized to Budesonide even in the absence of previous contact.

Similarly to other studies (9), this report confirms the validity of the Baek's hypothesis: some patients seem to recognize only the lateral chain of the steroid molecule with limited cross-reactivity, while others seem to recognize the skeletal structure of the molecule, with wide cross-reactivity. Some further differences

among these patients can be identified in the clinical history, in the way of sensitization and in test results.

This may suggest different management of patients: pattern 1 may have a few limitations related on the use of topical molecules only. For the two other patterns, due to the broad sensitization spectrum, it's necessary to find one or more alternative molecules to avoid the risk of systemic reactions.

As usual in drug allergy, the diagnostic workup depends on the way of administration of the drugs and the timing of onset of symptoms. The choice of the drugs to be tested depends on the clinical history and on the need of individual patient. Unfortunately, the diagnostic workup is not standardized yet. Moreover, the anti-inflammatory activity of CS can reduce the sensitivity, requiring different kinds of test (patch, intradermal and challenge test) to confirm the role of the drug as cause of reaction.

Conclusions

The results of this study show that in the clinical practice it's important to set up a workup tailored to the pattern of patients, evaluating multiple molecules for the assessment of the individual sensitization/tolerance profile. The classification of steroids in chemical groups appears to be of limited usefulness, due to the great variability of reactivity and sensitization of the patients. Moreover, it's not applicable to systemic corticosteroids and does not seem to be useful to predict SCD (10, 11).

The study has some limitations, mainly as a consequence of its retrospective design and the limited number of patients.

Nevertheless, studies like this could increase awareness of steroids hypersensitivity reactions and help clinicians on the management (11).

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Conflict of interests

The authors declare that they have no conflict of interests.

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Efficacy and quality of life assessment in the use of subcutaneous immunoglobulin treatment for children with primary immunodeficiency disorder

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Summary

Introduction. *Most patients with primary and secondary immunodeficiencies need regular intravenous immunoglobulin (IVIG) or subcutaneous immunoglobulin (SCIG) treatment. This study aimed to evaluate the serum IgG trough levels, frequency of mild and severe infections, frequency and duration of hospitalization, duration of absence of school, and quality of life in patients switching their IVIG therapy to SCIG administration.* ***Materials.*** *Twenty-nine patients with immunodeficiency on regular IVIG treatment and who agreed to receive SCIG treatment were included. Seven patients discontinued treatment after the first SCIG administration. We collected data regarding serum IgG levels, annual numbers of infections, hospital admissions, and adverse events prior to and following SCIG initiation. PedsQL tests such as scale total score (STS), physical health total score (PHTS), psychosocial health total score (PsyHTS), emotional functionality, social functionality, school/work problems score were calculated separately for all patients and their parents.* ***Results.*** *In twenty-two cases who were diagnosed as primary immunodeficiency, the most common indication for initiation of SCIG treatment was the long transfusion period of IVIG treatments and the difficulty of access to the hospital. No systemic side effects were noted except local redness, pain, and swelling on the injection site. The median IgG value was 588.9 mg/dl during IVIG treatment and 872 mg/dl one year after SCIG treatment. Annual frequency of infections and absence to school/work decreased significantly in the SCIG group while the annual number of hospitalizations and hospital stay time did not change significantly. There was a significant increase in the "quality of life" scores of the patients and their families.* ***Conclusions.*** *SCIG treatment provides ideal and protective immunoglobulin levels and offers the comfort of treatment in their home environment, thus increasing the patient's satisfaction and quality of life.*

Introduction

Primary immunodeficiency diseases (PID) predispose patients to recurrent respiratory, skin, and gastrointestinal infections and require Immunoglobulin (Ig) replacement treatment (1). Immunoglobulin replacement therapy has been a standard treatment for PID for more than 50 years since Bruton and his colleagues treated a patient with agammaglobulinemia by using intramuscular gammaglobulin (2). Ig replacement treatment can be administered intravenously every 3-4 weeks or subcutaneously

once every week. In 1991, Gardulf *et al.* demonstrated that subcutaneous immunoglobulin (SCIG) treatment was well tolerated and resulted in less systemic complications compared to intravenous immunoglobulin (IVIG) infusions in Swedish patients with PID (3). Recently, SCIG treatment has been widely used in Scandinavian countries, Europe, and other countries (4, 5). IVIG and SCIG treatment have different pharmacokinetics on serum IgG levels. IVIG treatment provides high peak IgG levels and low trough IgG levels, whereas SCIG treatment provides steady-state IgG levels (6). The two protocols have been tested

and proven to be effective, safe, and well-tolerated. SCIG treatment has become popular in recent years because of its self-administration at home, resulting in more stable serum IgG levels, better protection against infections, and fewer complications.

Chronicity of PID, delayed diagnosis, inadequate treatment and treatment side effects may effect the quality of life (QoL) of PID patients. QoL and the psychological impact of PID patients has been little studied in children. In a few previous studies, QoL was found to be poorer in PID patients than healthy controls (7, 8). Self infusion at home improves the QoL among patients, reduces travel time, and decreases healthcare system procedures (9, 10).

In this study, we aimed to evaluate the levels of Immunoglobulin G (IgG), frequency and severity of infections, frequency and duration of hospitalizations, time of absence from school/work and QoL in PID patients receiving IVIG and then SCIG treatment.

Patients and methods

Twenty-nine patients in follow-up with the diagnosis of primary immunodeficiency at Ege University Department of Pediatric Immunology and switched to SCIG treatment between May 2015 and August 2018 were included in this retrospective study. Patients eligible to participate in the study were diagnosed according to the European Society for Immunodeficiencies (ESID) criteria and were receiving IVIG regularly. These patients had some problems related to IVIG treatment in the hospital such as difficulties with venous access, lengthy and expensive travels to reach the hospital, and economic burden. The other inclusion criteria were their consent to receive SCIG treatment at home. Patients who were receiving IVIG treatment for neurological diseases (such as chronic inflammatory demyelinating polyradiculoneuropathy), skin diseases (such autoimmune bullous skin disorders) and hematological problems (such as chronic immune trombocytopenia) were excluded.

Twenty-two patients (75.8%) continued SCIG treatment, while seven patients (24.2%) left the study after the first application. Twenty-three males (79.3%), 6 females (21.7%) patients were included in the beginning but 18 males (81.8%), 4 females (18.2%) patients continued SCIG treatment. M/F ratio was 5/1, as usually seen in all PID patients (11). The diagnosis and results of molecular studies of all patients were listed in **table I**. Medical records of patients were reviewed retrospectively. Age at first IVIG therapy, gender, diagnosis, IVIG treatment dose and frequency, systemic and local IVIG complications, serum IgG levels and complications of diseases (bronchiectasis, chronic sinusitis, *etc.*), frequency of mild and severe bacterial infections, frequency and duration of hospitalization, duration of absence from school/work and complications of IVIG treatment were recorded. Pneumonia, bronchitis, otitis media, sinusitis, arthritis, and septicemia were accepted as severe infections.

Age at first SCIG treatment, reasons for switching to SCIG therapy, SCIG dose, injection site, infusion rate, local and systemic adverse reactions were recorded in the SCIG group. Besides, IgG serum levels were recorded at the end of the second week and the 2nd, 6th and 12th months. Patients were evaluated with the "quality of life test" Pediatric Quality of Life Inventory (PedsQL) on the 1st day, 6th and 12th month of SCIG treatment. After one year of SCIG treatment, infections and their severity, duration of hospitalization, absence from school/work, and serum IgG levels were evaluated.

Patients who switched to SCIG treatment received the first dose 15 days after the last IVIG treatment. SCIG treatment dose was calculated by dividing the IVIG treatment dose into weekly doses. It was applied once a week or every ten days. Immunoglobulin preparations at a concentration of 10%; KIOVIG® and GAMUNEX® solutions were used, because there was no other concentrated licensed/approved immunoglobulin preparation in our country. The training nurse performed the first application of SCIG treatment, and the second and third applications were accompanied by the training nurse in the hospital and applied by the patient or parent. The patient/parent administered subsequent doses at home. SCIG treatment was applied to all patients with a soft set butterfly needle at a distance of 2-3 cm from the abdomen to the umbilicus. The total dose is divided into two equal doses from both sides of the abdomen per region; it was applied simultaneously with the manual rapid infusion technique with the help of injectors of maximum 25 cc for patients less than 40 kg, maximum 35 cc for patients more than 40 kg, with a speed of 2 ml/min. A maximum of 10 g of immunoglobulin was applied in one application. No premedication was applied before infusions. Infusion times, application site and adverse effects were recorded.

Statistical analyzes were performed with SPSS 22.0 (IBM SPSS Statistics, Chicago, IL) program. Descriptive statistics incuded mean ± standard deviation or median (minimum-maximum) for continuous variables and nominal variables as the number of cases and (%). Chi-Square test for categorical data comparisons between groups, Kruskal-Wallis and Mann-Whitney U tests were used for comparison of numerical and ordinal data. Mann-Whitney U test was used to evaluate the quality of life data. A p-value of < 0.05 was considered statistically significant.

Results

Diagnostic distribution of patients with primary immunodeficiency were listed in **table I**, while common variable immunodeficiency (CVID) (n = 13; 44.8%) and X-linked agammaglobulinemia (Bruton disease) (n = 7; 24.1%) were the most common diseases. Five were diagnosed as Bruton's disease and two as CVID of the seven cases who preferred to discontinue SCIG treatment. The reasons to leave the treatment were mostly the

concerns about injecting a drug to themselves or their children. Statistical analyses were performed on 22 patients who continued treatment. The age of the patients was significantly higher in the SCIG group (**table II**). The reasons for choosing SCIG

treatment were difficulty for reaching the vascular access in 14 cases (63.6%), frequent transfusion needs in 16 cases (72.7%), long IVIG infusion time in 19 cases (86.3%), difficulty in accessing the hospital in 18 cases (81.8%). Five cases (22.7%) had

Table I - Age, gender, molecular genetical findings and diagnosis of the study group.

Patient No	Age/ Year	Gender	Diagnosis	Molecular and Genetics Diagnosis
1	23	M	CVID	Not finalized yet
2	8	M	Combined immune deficiency	*PLCG2 gene*; heterozygous p.ser718Arg mutation was found
3	7.5	M	TTC37 mutation	*TTC37 gene*: c.2210 T > C p.val 737A1a homozygous mutation was found
4	32	M	Bruton disease	*BTK gene*; Glutamik asit 76 to stop (guanine358timin)
5	5	M	Bruton disease	*BTK gene*: Arg5256/g hemizygous mutation was found
6	29	M	X'linked Lymphoproliferate disease	*SH2D1A gene*; c.163C > T R55Ter hemizygous mutation was found
7	28	M	CVID	*TNFSF13B gene* (TACI); Pro97Pro ve Ser277Ser synonymous amino acid mutations were found
8	14	F	CVID	Mutation was not found in TNGS (Targeted Next Generation sequencing test)
9	16	M	CVID	-*TNFSF13 gene* (APRIL); Asn96Ser mutation was found -*TNFSF13B gene* (TACI): Thr27Thr synonymous amino acid mutation was found
10	11	M	CVID	Not finalized yet
11	11	M	Di-George syndrome	FISH; 22q11 deletion was found
12	22	M	Activated PI3K-delta syndrome(APDS)	*PIK3CD gene*; c.970C > G p. Arg324Gly mutation was found
13	26	M	CVID	*TNFSF13B gene* TACI: heterozygous c.204-205 insA (p.L69TfsX12) mutation was found
14	11	M	LRBA deficiency	*LRBA gene*: c.2496C > A, p.cys382Ter(P.c832*) mutation was found
15	13	M	LRBA deficiency	*LRBA gene*: c.2496C > A, p.cys382Ter(P.c832*) mutation was found
16	12	F	HiperIgE syndrome STAT 3 defect	*STAT3 gene*: c114C > T,p.Arg382Tryp mutationu was found
17	7.5	M	CVID	Mutation was not found in TNGS (Targeted Next Generation sequencing test)
18	19	M	CVID	-*TNFSF13 gene* (APRIL): Asn96Ser mutation was found -*TNFSF13B gene* (TACI) : Thr27Thr synonymous amino acid mutation was found
19	12	F	CVID	Not finalized yet
20	19	F	HiperIgE syndrome STAT 3 defect	*STAT3 gene*: in SH2 domain tirozin 657 changed to sistein, 1970A > G, Y657C
21	10	M	CVID	*TNFRSF13B gene,* TACI; heterozygous P.CYS 193 Ter generating stop codon. Predefined mutation disease factor with OD transition
22	10	M	CVID	Not finalized yet
*Patients leaving SCIG treatment (n = 7)				
23	9	M	Bruton disease	*BTK gene: Glutamik asit 76 to stop (guanine358timin)*
24	5	F	CVID	*PLCG2 gene*; heterozygous p.ser718Arg mutation was found
25	8	M	Bruton disease	*BTK gene*; c.36G > C p.Lys12Asn hemizygous mutation was found
26	3	F	Bruton disease	*BTK gene*; c.36G > C p.Lys12Asn hemizygous mutationu was found
27	1	M	CVID	Not finalized yet
28	8	M	Bruton disease	*BTK* gene; c.493T > G (p.Cys165Gly) hemizygous mutation was found
29	6	M	Bruton disease	*BTK* gene; c.493T > G (p.Cys165Gly) hemizygous mutation was found

M: male; F: female.

some personal preferences. None of the patients preferred SCIG treatment due to the serious side effects of IVIG treatment.

There were some complications related to the underlying diseases in 21 patients. No complication was recorded for a CVID patient. Seven cases had only one complication, while the other 14 had two or more complications. Growth retardation (n = 11; 50%), bronchiectasis (n = 12; 54.5%), chronic sinusitis (n = 3; 13.6%), hepatomegaly (n = 2; 9.0%), splenomegaly (n = 4; 18.0%), chronic diarrhea (n = 6; 27%), autoimmunity (n = 2; 9.0%), and some other complications (n =12; 54.5%) were observed.

IVIG group

Twenty-two patients in the study group received a median of 0.5 (0.17-1) g/kg IVIG treatment in the last year before SCIG treatment. The median time between the two treatments was 45 (21-75) days. The median IgG value was 588.9 (136-1938) mg/dl. Two patients with Hyper IgE syndrome and one patient with SH2D1A defect had hypergammaglobulinemia, so the mean IgG value was found to be slightly higher than age-related normal values (12). The median annual frequency of infections was 4 (1-12) times, severe bacterial infection was 0.5 (0-3) times, and absence to school/work was found to be 12 (0-90) days. In IVIG study group, 9 (37.5%) of 24 cases had rapid side effects such as fever and rash, while 15 cases (64.5%) had no side effects. None of the cases had serious side effects due to IVIG treatment.

SCIG group

When switching to SCIG treatment, the monthly immunoglobulin dose received by the patients was divided into four, and a median of 0.11 g/kg/week (0.06-0.41) immunoglobulin was administered subcutaneously. The average initial infusion time for SCIG therapy was 22.4 minutes (4-50 minutes). In all cases, the first three applications were performed by the training nurse, subsequent practices were performed by parents (n = 15; 68.1%) and by patients (n = 7; 31.9%). 10% solutions were used in all cases for SCIG therapy: GAMUNEX® was preferred in 3 cases (13.6%), and KIOVIG® in 19 cases (86.4%). Local side effects (redness, swelling and mild pain) were observed in 20 cases (90.9%). Systemic side effect due to SCIG treatment was not observed in any of the cases. Trough IgG levels after SCIG treatment were shown in **figure 1**. When IVIG treatment IgG trough levels were compared with SCIG trough levels at 12th months, a statistically significant difference was found (p = 0.000) (**table II**). Annual frequency of infections, and absence to school/work decreased significantly in SCIG group while annual severe bacterial infections, number of hospitalizations, and hospital stay time did not change significantly (**table II**).

Figure 1 - *Trough IgG levels after SCIG treatment.*
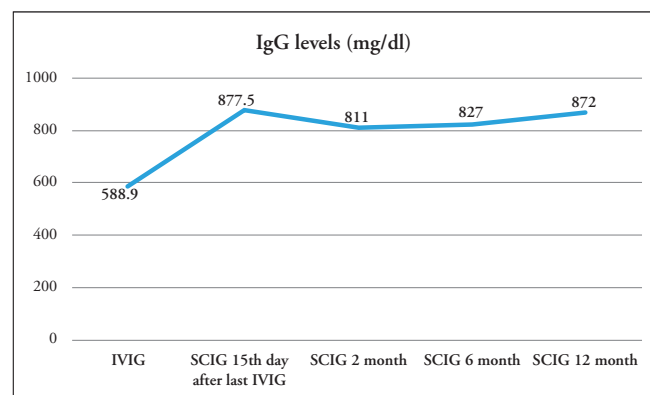
Table II - *Clinical and laboratory features of patients during IVIG and SCIG treatments.*

Clinical and Laboratory Features	During IVIG Treatment	During SCIG Treatment	P values
Age (years) median (min-max)	5.5 (1-16)	11 (4-30)	0.000
Immunoglobulin dosage in the last year median (min-max)	0.5 (0.17-1) (g/kg/month)	0.11 (0.06-0.41) (g/kg/week)	*
Time (days) between two immunoglobulin therapy median (min-max)	45 (21-75)	7 (7-10)	*
IgG (mg/dl) median (min-max)	588.9 (136-1938)	872 (411-2030)	0.000
Annual frequency of infections (times) median (min-max)	4 (1-12)	1 (0-8)	0.000
Annual severe bacterial infections (times) median (min-max)	0.5 (0-3)	0 (0-4)	0.234
Number of hospitalizations in a year median (min-max)	0 (0-3)	0 (0-7)	0.231
Annual hospital stay time (days) median (min-max)	0 (0-36)	0 (0-48)	0.960
Absence to school/work days/year median (min-max)	12 (0-90)	3 (0-50)	0.002

*Parameters are not suitable for comparison.

Details of PedsQL tests such as scale total score (STS), physical health total score (PHTS), psychosocial health total score (PsyHTS), emotional functionality, social functionality, school/work problems score which was carried out separately for 24 patients and their parents are shown in **figures 2, 3**. These scores were determined three times during SCIG treatment and it was observed that the point value increased in each group. A statistically significant difference in the PedsQL scores ($p < 0.05$) was found when the 6th month and 12th month were results compared with the first day.

Discussion

IVIG and SCIG have been repeatedly shown to reduce mild and severe infections and increase in serum IgG concentrations and are thus both approved methods of Ig replacement (13). In recent decades SCIG has been used increasingly, with some data showing increased IgG levels, improved patient quality of life indicators and decreased overall cost to the healthcare system when compared to IVIG (13).

In the follow-up of immunoglobulin treatments, the frequency of mild and severe infections, time lost from school and work, and serum trough IgG levels are critical indicators to determine the efficacy of the treatment. Although the target serum IgG level is stated as at least 500 mg/dl in some publications, it has been observed that IgG dosage needs to be adjusted on an individual basis to prevent infections (14, 15). According to the data obtained from recent studies, the recommended average serum IgG value has to be 700-800 mg/dl (16). In a prospective study (n = 60) by Gardulf et al. (17), SCIG therapy for the first 15 weeks of treatment is defined as 'washout period' and 16-43 weeks as "efficacy period". In the same study, while the mean serum IgG level was 780 mg/dl at the beginning of the treatment in the pediatric age group, the mean of the efficacy

phase increased to 920 mg/dl. In a study by Chapel et al. (18) (n = 40), it was reported that the mean serum trough value of IVIG treatment was 780-840 mg/dl while it was 800-910 mg/dl in SCIG treatment. In the study by Bezrodnik et al. (19) (n = 15), the mean serum IgG value was 960 mg/dl before SCIG treatment, while it was reported to be 1309 mg/dl at 24th week and 1230 mg/dl at 36th week. In the study of Aydıner et al. (20) (n:16), mean serum IgG 976 mg/dl increased to 1025 mg/dl after treatment, but no statistically significant difference was found. In our study, while the median trough IgG levels were 588.9 mg/dl before SCIG treatment, it was found to be 827 mg/dl in the 6th month of the treatment and 872 mg/dl in the 12th month, showing that they reached the recommended level. Patients with PID most frequently present with sinopulmonary findings. In previous reports, the number of mild infections, such as upper respiratory tract infections, decreased significantly under SCIG treatment. However, when the authors compared the frequency of acute severe bacterial infections between the two treatments, it was shown that there was no statistically significant difference (17, 21). In our study, we found a significant decrease in the annual frequency of infections in the SCIG group, while annual severe bacterial infections did not change significantly (**table II**). Our findings on the frequency of infections were consistent with the results in the literature.

The patient or the parent can administer SCIG treatment at home, and this treatment reduces dependency on the hospital and days off school and work due to illness (22). Besides, it does not require vascular access; this highly increases the life quality of the patients. In our study, absence from school/work decreased significantly in the SCIG group (median three days) compared to the IVIG group (median 12 days) (**table II**). How-

Figure 2 - Patients' PedsQL test results.
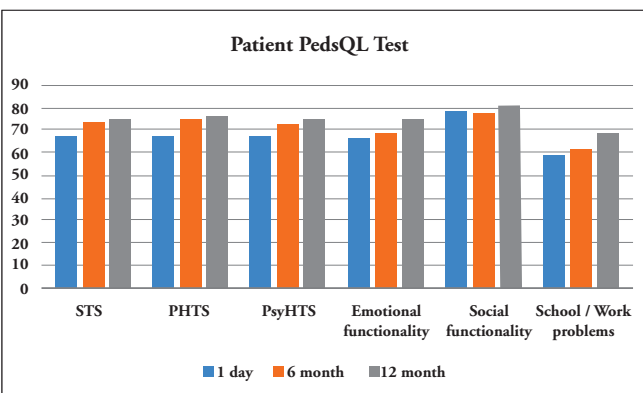
STS: scale total score; PHTS: physical health total score; PsyHTS: psychosocial health total score.

Figure 3 - Parents' PedsQL test results.
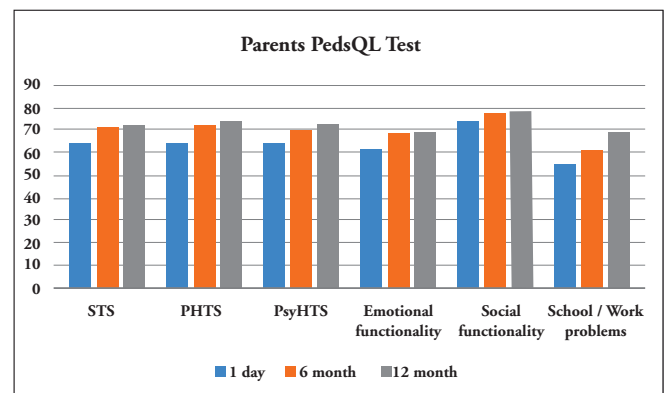
STS: scale total score; PHTS: physical health total score; PsyHTS: psychosocial health total score.

ever, the number of hospitalizations or duration of stay in hospital did not change significantly between the groups (**table II**). Health-related Qol in PID patients was measured in limited studies. Ateinia *et al.* (8) measured QoL using PedsQL and SF-36 questionnaries in 70 PID patients. Patients expressed reduced scores in some mental and physical components (8). Patients with long follow-up time had higher scores in mental components and still low scores in physical components (8). Titman *et al.* (7) collected both parental and child ratings using standardised questionnaries such as PedsQL and SDQ in PID patients. Nineteen children and 43 parents completed the SDQ and 39 children and 43 parents completed the PedsQL. Higher rates of psychological difficulties were found in PID cases when compared with healthy controls (7). QoL was also poorer than in healthy controls. These QoL studies were also performed after switching IVIG treatments to SCIG and SCIG treatment has been found to improve QoL (23). In the study by Vultaggio *et al.* (24) (n = 50); while there was no significant difference in physical, daily activity and total health scores, there was a significant improvement in the QoL index. In a study by Gardulf *et al.* (25), the author emphasized the increase in the autonomy and QoL of the patient's self-application at home and being connected to others. In our study, the QoL including physical health score, psychological health score and total health score, emotional functionality, social functionality, school/work life was found to be increased in both patients and families. It has been reported that side effects during IVIG therapy were observed in 20% of cases (26). Although most of these are temporary and non-serious side effects, 2-6% of them are serious (27). Side effects due to IVIG treatment are observed in 3 different groups; rapid side effects (within 6 hours after infusion), delayed side effects (within 6 hours, 1 week after infusion) and late side effects (weeks and months later) (28). In our study, 9 (37.5%) of 24 cases had rapid side effects such as fever and rash, while 15 cases (64.5%) had no side effects. None of the cases in study group had serious side effects due to IVIG treatment. While IVIG directly enters the blood, SCIG first passes into the lymphatic circulation and then into the bloodstream with the thoracic duct. Therefore, in SCIG, the slow transition to systemic circulation increases the tolerability of SCIG and reduces the possibility of systemic side effects (29). Although systemic side effects due to SCIG treatment are rare, local side effects such as rash, itching, and mild pain are observed. In the study by Chapel *et al.* (18) (n = 30), local side effects such as pain, redness and swelling were observed in all cases, while systemic side effects were observed only in 3.3%. In another study by Bezrodrik *et al.* (19) (n =15), 12 cases reported swelling, redness, pain and itching, while no systemic side effects were reported. In the study by Aydıner *et al.* (20) , 6% of local side effects (redness itching, swelling) were reported, while systemic side effects were not reported. In our study, while more local side effects (n = 22) (91.6%) (redness, swelling, mild pain) due to SCIG application developed, no systemic side

effects were observed, similar to other studies. It is thought that the presence of more local side effects is due to the rapid push and short SCIG infusion time (27.4 ± 17.7 minutes).

SCIG treatment has many advantages for patients who do not want to be hospitalized. These patients have difficulty for the venous vascular access or they cannot tolerate IVIG treatment or they do not want to stay in the hospital for a long time and want a more flexible treatment program (30). It is preferred also in terms of increasing the QoL of the patients who live far away from the hospital and who have busy work and school life and travel frequently (31). In the studies by Chouksey *et al.* (32) and Aydıner *et al.* (33); it has been reported that the most common reason to prefer SCIG treatment is difficulty in reaching the vascular access and side effects due to IVIG treatment. On the other hand, some patients may be discouraged from switching from IVIG to SCIG on account of perceived inconvenience, concerns about adverse effects at home and fear of needle sticks (33). In our study, the most frequent reasons were the long IVIG infusion period in the hospital and the difficulty to travel to the hospital. This situation can be explained by the fact that the pediatric immunology clinic of our hospital is one of the largest pediatric immunology clinics in the Aegean region and the number of patients living far away is very high.

The recommended starting dose for the treatment of Immunoglobulin G is for IVIG; 0.4-0.6 g/kg in 3 or 4 week intervals, 0.1-1.15g /kg week for SCIG (29). There are differences between Europe and America in terms of dose applied in the transition from IVIG treatment to SCIG treatment. In European countries, the amount of IgG given monthly is divided into 4-week doses at the same rate and administered subcutaneously (34). The American Food and Drug Administration (FDA) recommends a weekly dose calculation by multiplying the current dose by changing the coefficients depending on the product concentration (× 1.37 for 16% and 1.53 for 20% products) (27). In our study, the median IVIG dose was 0.5 g/kg and it was similar to the literature. The SCIG doses taken by the patients were administered by dividing them evenly into weekly doses. In our study, the median SCIG dose was 0.11 g/kg/week and it was consistent with the literature data.

Application of SCIG at high volume and speed is known to increase the development of local side effects such as swelling, redness and pain. Recent, studies have shown that the application of 35ml/h does not create any more side effects than the application of 20 ml/h (35). In our study, no more than 100 ml was administered once and no more than 50 ml per region was administered in 18 minutes. The average initial infusion time was 22.4 minutes (4-50 minutes), and at the end of one year, the average infusion time extended to 26.7 minutes (12-75 minutes). Similar to the work of Vultaggio *et al.* (34), in our study local side effects were observed to decrease when the infusion time increased in cases with ongoing and home applications.

There are two limitations of this study. First, the study is limited by the small cohort size and secondly by short term study dura-

tion (3 years). If we had longer follow-up time, we could decide about the benefits of SCIG therapy on the disease complications occured before and following the improvements in patients' disease complications, quality of life indexes might be much better.

Conclusions

In conclusion, SCIG treatment can be preferred in cases followed-up with the diagnosis of primary and secondary immunodeficiency due to difficulties in access to the venous vein, serious side effects due to IVIG, difficulty in reaching to hospital and also for the patients who desire a more flexible lifestyle. In addition, in patients using IVIG and with very low serum trough IgG value, SCIG is a good treatment option. We suggest that administration of smaller doses in shorter periods increases the serum trough IgG level and provides a more stable level, which is due to pharmacokinetic and dynamic differences. SCIG treatment reduces abcence from school/ work and provides time for people to travel comfortably and to move freely, due to the decrease in the number of infections and their application at home resulting an increase in the quality of life of both the patients and the families.

Conflict of interests

The authors declare that they have no conflict of interests.

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Interference of *Dermatophagoides pteronyssinus* sensitization in grass pollen allergy

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Summary

Background. *Climate conditions in the northwest of Spain are from the rest of the country, and the pollen sensitisation rates and allergens involved are different. The present study aimed to investigate the sensitisation profile of patients with grass pollen allergy and the interference of other sensitisations in respiratory symptoms.* **Methods.** *A total of 959 Spanish patients with seasonal respiratory symptoms and a positive skin prick test (SPT) to Phleum pratense pollen were studied. Patients were classified as having rhinitis and/or bronchial asthma. A battery of SPTs, including common weeds and tree pollens, profilin, polcalcin, moulds, Dermatophagoides pteronyssinus, Lepidoglyphus destructor, and cat and dog dander were performed. Serum specific IgE (sIgE) to Phl p 1 and Phl p 5, adding sIgE to Phl p 7, Phl p 12 and house dust mites (HDMs) or other pollens in selected cases were measured.* **Results.** *The majority (89.8%) of the patients were polysensitised according to SPT. HDM co-sensitisation was the most prevalent (62.3%). Profilin and polcalcin rendered a positive result in 25.9% and 18.7% of the patients, respectively. A higher proportion of patients recognized sIgE to Phl p 1 (88.7%) with respect to Phl p 5 (59%). Phl p 1-sIgE levels were higher than Phl p 5-sIgE levels, and no differences were found in patients with rhinitis and/or asthma. However, total serum IgE was higher in patients with asthma. Multivariate regression analyses revealed that only sIgE to Dermatophagoides pteronyssinus (after adjusting by sIgE to Phl p 1, Phl p 5 and Lepidoglyphus destructor) was associated with a greater risk of asthma.* **Conclusions.** *Phl p 1 is the most relevant allergen in patients with grass pollen allergy in the northwest of Spain. Sensitisation rates against panallergens are low. Even in patients with grass pollen allergy, HDM sensitisation plays a relevant role in asthma.*

Introduction

The northwest of Spain is a wet region with mild temperatures similar to Northern and Central Europe in terms of pollination (1-3). In our health care area, *gramineae* (*Poaceae*) is by far the most relevant pollen from an allergological point of view (1, 4). Other relevant pollens are those from the *Betula* and *Parietaria* genus. However, house dust mites (HDMs) constitute the major source of aeroallergens and are the leading cause of allergic rhinitis and allergic asthma in this region (5-7). In a previous prospective study, we have shown that the majority of patients with HDM

allergy without any other sensitisation recall seasonal changes in symptoms (8), making it difficult to differentiate seasonal pollen symptoms from HDM allergies in polysensitised patients.

Over the last 3 decades, the emergence of molecular diagnosis has been considered an essential tool for the diagnosis and treatment of allergic diseases (9-14). However, molecular diagnoses must be complemented with a clinical approach and a profound knowledge of the main allergens in a specific area. Regarding grass pollen allergy, the grass species commonly used as a model is *Phleum pratense*, in which more than 13 different allergens have been identified (15). Although sensitisation rates for every

allergen component vary from region to region, it has been accepted that Phl p 1 and Phl p 5 are major allergens from *Phleum pratense*, found in 95% and 65%-85% of patients, respectively (14, 16). The presence of specific immunoglobulin E (sIgE) against Phl p 1 and Phl p 5 is considered to indicate genuine sensitisation to grass pollen. In contrast, profilin (Phl p 7) and polcalcin (Phl p 12), widely distributed among pollens and vegetables, are known as panallergens and could be considered as markers of disease progression, polysensitisation, and/or provoking type II food allergy symptoms (4, 17). Their sensitisation rate varies from 10% to 20% (4) in Spanish populations but reaches 30% when including patients from other European countries (18, 19). The present study aimed to investigate the sensitisation profile of our patients with grass allergy and the interference of other sensitisation in the interpretation of the clinical pattern, the type of respiratory symptoms and/or food allergy.

Patients and methods

Study population and design

This was a cross-sectional study that enrolled 959 adults with seasonal respiratory symptoms and/or symptoms compatible with oral allergy syndrome (OAS) and a positive skin prick test (SPT) to *Phleum pratense* pollen who first attended our reference University Hospital Allergy Department in northwest Spain from January 2007 to December 2011. Patients were referred either from Primary Care or from the Ear, Nose and Throat Department. The hospital covers an area of approximately 500,000 people; nearly 90,000 live in the city of Santiago de Compostela, and the remainder live in primarily rural areas. The median age was 26 years (interquartile range (IQR), 17-35 years) and 513 (53.5%) were female. The local weather is warm and humid, and as previously stated, grass pollen is the most relevant pollen in the area (4) even though HDM sensitisation is higher than pollen sensitisation (5). Patients were classified according to their clinical histories and spirometry values (FEV1 increase greater than 12% and 200 mL after bronchodilator test) as having rhinitis or rhinoconjunctivitis (n = 553, 57.7%), asthma (n = 17, 1.8%) or both (n = 377, 39.3%). The remaining 11 (1.1%) patients did not meet the clinical criteria for respiratory allergy despite having a positive SPT. A physician-administered questionnaire was completed for every patient, including data on symptoms after eating vegetables and fruits.

Complementary studies

SPTs were performed on the volar surface of the forearm using a battery of common allergens in the region, including *Phleum pratense, Plantago lanceolata, Parietaria judaica, Artemisia vulgaris, Betula alba, Alnus glutinosa, Olea europaea, Fraxinus excelsior, Quercus robur, Platanus acerifolia,* purified natural date palm profilin

(Pho d 2 50 μg/mL), date palm polcalcin, *Dermatophagoides pteronyssinus, Lepidoglyphus destructor, Alternaria alternata, Aspergillus fumigatus* and cat and dog dander (ALK-Abelló, Madrid, Spain). Total serum IgE was measured by latex-enhanced nephelometry in a BN-II analyser (Siemens, Germany). Allergen-sIgE was measured by the Immuno-CAP-250™ system (Thermo-Fisher Scientific, Sweden) and included sIgE against Phl p 1 and Phl p 5 as molecular markers of grass pollen sensitisation and sIgE against house dust mites or other pollens if a positive SPT was found with presumably clinical relevance. In patients with a positive SPT against profilin and/or polcalcin and patients with symptoms of OAS, food allergy or positive SPT against pollen allergens different from grasses, sIgE to Phl p 7 and/or Phl p 12 were also measured. Following the manufacturer's instructions, sIgE levels ≥ 0.1 kU$_A$/L were deemed positive; for analyses, however, the classic 0.35 kU$_A$/L threshold level was used.

Statistical analyses

Continuous variables were expressed as median and IQR. Categorical variables were expressed as absolute numbers and percentages. Spearman´s rank tests were employed for evaluation of correlation of continuous variables. Logistic regression analysis was employed to estimate the association between serum specific IgEs and asthma. Statistical significance was accepted at p < 0.05. All analyses were developed using the Statistical Package for Social Sciences (IBM SPSS, v. 19).

Ethics

The study was approved by the Institutional Review Board of Complejo Hospitalario Universitario de Santiago de Compostela and complied with the recommendations of the Declaration of Helsinki.

Results

According to our inclusion criteria, all patients presented a positive SPT to *Phleum pratense*, and the majority of respiratory symptoms could be classified as upper and/or lower airway disease (see Methods). Only 121 (12.6%) patients reported OAS symptoms when eating vegetables or fruits. More than 50% of the patients reported mild respiratory symptoms at home related to dust exposure.

The majority (844, 89.8%) of our patients presented a positive SPT to at least one allergen different from grass pollen. HDM sensitisation was detected in more than 50% of this population (a higher sensitisation rate against *Dermatophagoides pteronyssinus* than against *Lepidoglyphus destructor*, 585 (62.3%) *vs* 408 (43.5%), respectively). The highest sensitisation rates against other pollens were 30.8%, 25.2% and 23.5% against *Betula alba, Olea europaea* and *Parietaria judaica*, respectively. SPT

against profilin and polcalcin rendered a positive result in 249 (25.9%) and 180 (18.7%), respectively.

Specific IgE to Phl p 1 and Phl p 5 was available for 904 and 913 patients, respectively. Regarding grass pollen sensitisation, a higher proportion of patients had sIgE to Phl p 1 (88.7%) with respect to Phl p 5 (59%). Concentrations of Phl p 1-sIgE were higher than those of Phl p 1-sIgE (median, 7.24 kU$_A$/L (IQR 1.26-22.87 kU$_A$/L) vs median 2.09 kU$_A$/L (IQR 0.01-17.6 kU$_A$/L), respectively). A significant correlation was found between Phl p1-sIgE and Phl p 5-sIgE (R = 0.7, P < 0.001) (**figure 1**).

Serum sIgE against profilin (Phl p 12) was measured in 471 patients (443 because of more than one pollen sensitisation and 249 because of a positive SPT against profilin). Also, sIgE against polcalcin (Phl p 7) was measured in 400 patients (180 with a positive SPT). In both cases, sIgE levels were low (Phl p 7-sIgE median, 0.01 kU$_A$/L (IQR 0.01-0.07 kU$_A$/L) and Phl p 12-sIgE median 0.19 kU$_A$/L (IQR 0.02-1.23 kU$_A$/L). The concordance of the SPT extracts of profilin and polcalcin and sIgE to the corresponding panallergens was evaluated. A higher diagnostic value was observed for profilin SPT (positive concordance 85.5%; negative concordance 77.98%) than for polcalcin SPT (positive concordance 42.8%; negative concordance 93.6%) (**table I**). Only 60.1% of patients reporting OAS presented a positive result to Phl p 12 in serum.

Concentrations of sIgE to Phl p 1 and sIgE to Phl p 5 did not differ according to the patients' clinical diagnoses, rhinitis and/or asthma (**table II**). However, total serum IgE was significantly higher in patients with asthma with or without concomitant rhinoconjunctivitis (**table II** and **figure 2**). The comparison of total IgE among patients with other pollen sensitisations yielded no differences (data not shown). Nevertheless, patients with HDM sensitisation, especially those sensitised to *Dermatophagoides pteronyssinus*, presented higher levels of both total IgE and also sIgE to *Dermatophagoides pteronyssinus* and *Lepidoglyphus destructor* in patients with asthma (**table II** and **figure 3**). In fact, multivariate regression analyses revealed that only sIgE to *Dermatophagoides pteronyssinus* (after adjusting by sIgE to Phl p 1, Phl p 5, and *Lepidoglyphus destructor*) was significantly associated with a greater risk of asthma (**table II**); the same result was obtained even after adding sIgE to Phl p 7 and sIgE to Phl p 12 as variables into the equation. The risk of asthma significantly increased as the level of sIgE to *Dermatophagoides pteronyssinus* increased (**figure 4**).

Discussion

The results of our study suggest that even in patients with grass pollen allergy, the weight of HDM sensitisation in the clinical expression is high in the northwest of Spain.

Regarding SPT results, almost 90% of our patients with grass pollen allergy were polysensitised. However, the most frequent sensitisation found was to HDM, given 62% of patients showed

Figure 1 - *Scatterplots representing the relationship of serum specific IgE to Phl p 1 with specific IgE to Phl p 5.*
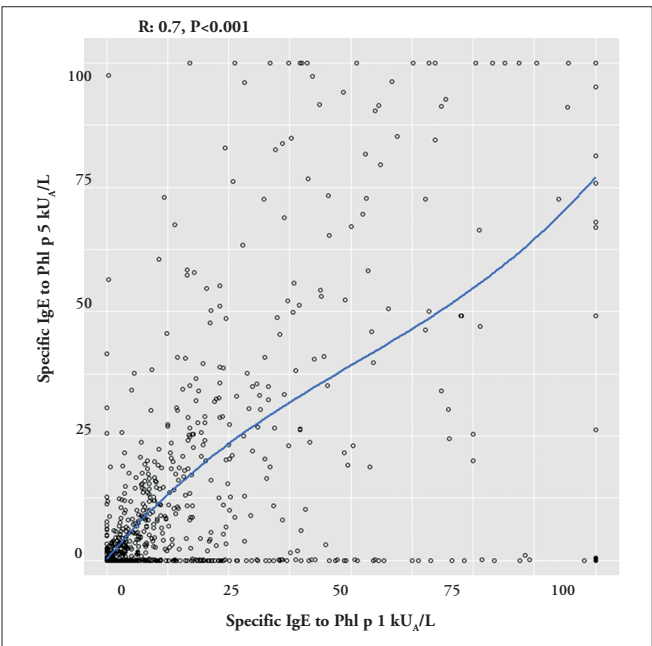

Figure 2 - *Total serum IgE levels (IU/mL) in patients with grass pollen allergy according to the clinical diagnosis of rhinitis and/or bronchial asthma.*
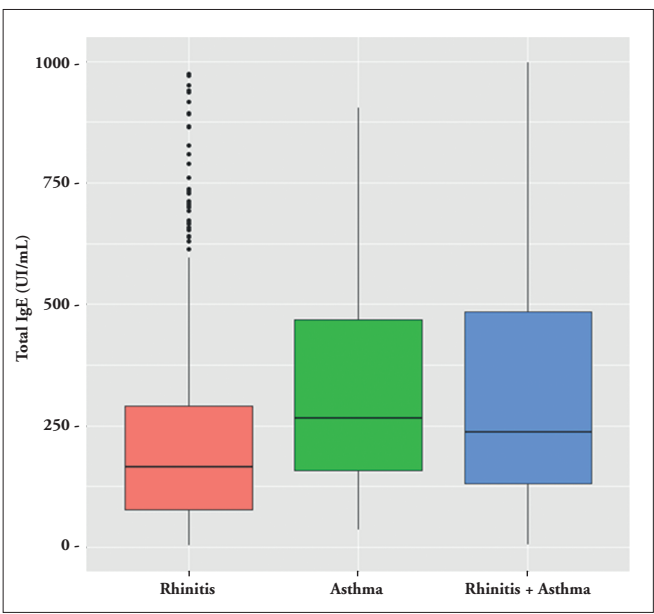
Figure 3 - *Serum specific IgE levels to Dermatophagoides pteronyssinus and Lepidglyphus destructor in patients with grass pollen allergy according to the clinical diagnosis of rhinitis with and/or bronchial asthma.*
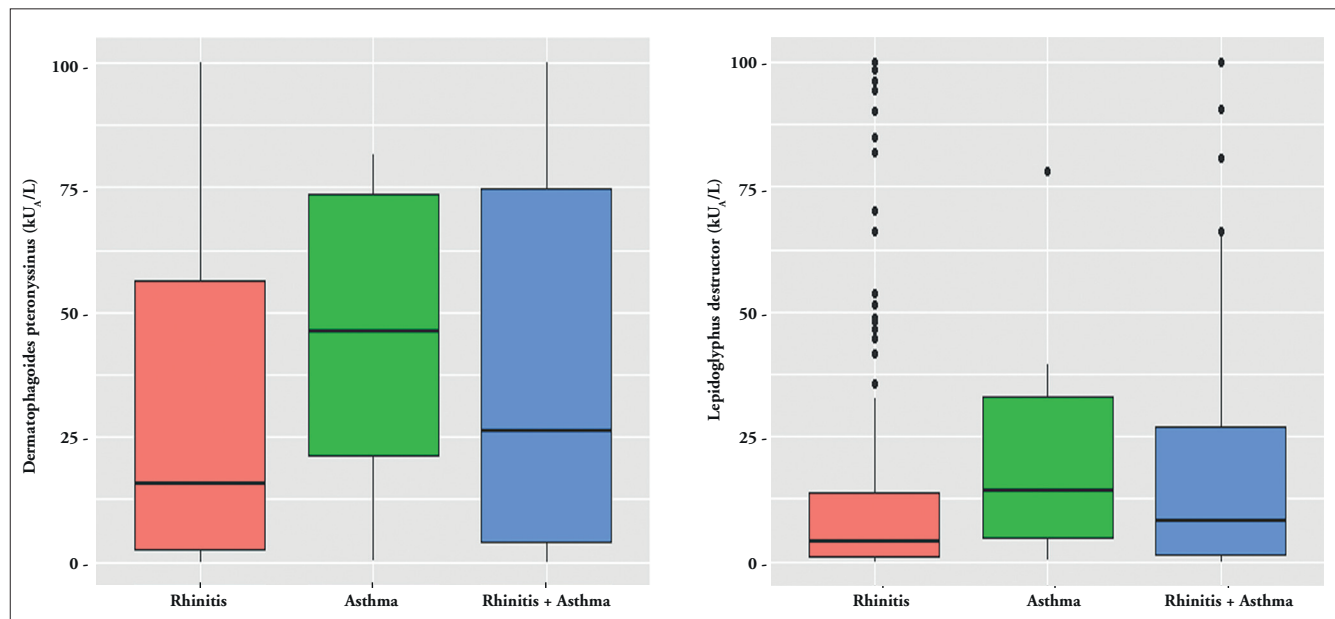

Table I - *sIgE to Phl p 1, Phl p 5, Dermatophagoides pteronyssinus, and Lepidoglyphus destructor and total serum IgE in patients with grass pollen allergy patients with or without bronchial asthma.*
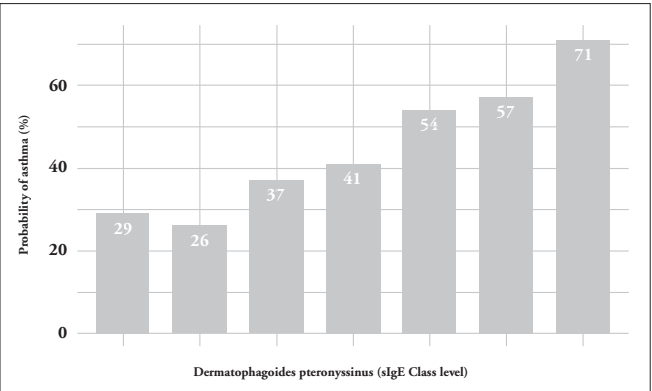
Allergen	Rhinitis (n = 553)	Asthma (n = 17)	Rhinitis + Asthma (n = 377)
Phl p 1	6.6 (1.0-21.7) kU$_A$/L (n = 525)	8.3 (1.6-13.3) kU$_A$/L (n = 14)	7.8 (1.7-23.9) kU$_A$/L (n = 355)
Phl p 5	1.5 (0.0-14.7) kU$_A$/L (n = 527)	5.7 (0.0-17.3) kU$_A$/L (n = 15)	2.4 (0.0-22.5) kU$_A$/L (n = 362)
Dermatophagoides pteronyssinus	30.1 (6.8-67.3) kU$_A$/L (n = 292)	42.7 (19.6-78.9) kU$_A$/L (n = 11)	44.2 (12.7-99.9) kU$_A$/L (n = 214)
Lepidoglyphus destructor	4.3 (1.1-14.1) kU$_A$/L (n = 179)	14.1 (3.4-37.2) kU$_A$/L (n = 8)	8.5 (1.6-27.2) kU$_A$/L (n = 146)
Total IgE	183 (90-399) UI/mL*	282 (157-533) UI/mL	289 (141-725) UI/mL

sIgE: specific IgE. Figures are shown as median (IQR). Cases with sIgE above the analytical limit (100 kUA/L) were deemed to have 100 kUA/L. (*) Patients with rhinitis presented lower levels of total serum IgE than patients with asthma (with or without concomitant rhinitis) ($p < 0.001$).

a positive response to *Dermatophagoides pteronyssinus*, an allergen commonly found in this region (5-7, 20). Only 47.1% of patients presented positive SPTs to pollens different from grass pollen, a lower prevalence than the 70% reported in other regions in Spain (21). The prevalence of sensitisation to profilin and polcalcin was similar to that reported by other authors in Spain (22-25); however, the prevalence of OAS in our population was at least 3-fold lower than that reported by the same authors. A pos-

sible explanation for the low rate of OAS in our patients could be a lower sIgE level against Phl p 7 and Phl p 12 in our patients, perhaps indicating a lower environmental exposure (17). Taking into account the low rate of OAS in our population, the concordance between sIgE determination and a positive SPT to profilin and that only 60% of patients with OAS presented positive sIgE to Phl p 12, we suggest that SPT is enough for the diagnoses (4).

Figure 4 - *Prevalence of asthma in grass pollen allergic patients with co-sensitization against HDM (Dermatophagoides pteronyssinus) in relation to the level of specific IgE to Dermatophagoides pteronyssinus scored in classes: Class 0: no detection of sIgE < 0.35kUA/L; Class 1: 0.36-0.70 kUA/L; Class 2: 0.71-3.50 kUA/L; Class 3: 3.51-17.5 kUA/L. Class 4: 17.51-50 kUA/L; Class 5: 50-100 kUA/L; Class 6: > 100 kUA/L.*

Table II - *Analysis of variables associated with bronchial asthma.*

	Coefficient (B)	SE (B)	P-value
sIgE to Phl p 1	0.002	0.008	0.753
sIgE to Phl p 5	0.007	0.008	0.346
sIgE to *Dermatophagoides pteronyssinus*	0.017	0.004	< 0.001
sIgE to *Lepidoglyphus destructor*	0.004	0.007	0.588

Multiple regression analysis. All selected variables entered the equation. B: slope of the regression model. SE: standard error.

With respect to the sensitisation profile against grass pollen, Phl p 1 is both more frequently detected and has higher sIgE levels, as has been established in previous series in Europe (4, 11, 26). A few studies have noted the relationship between sensitisation profile and the severity of the respiratory disease or its clinical expression. Savi et al had studied 140 patients with grass pollen allergy and found that patients without asthma presented low values of sIgE to Phl p 5, suggesting that high levels of sIgE to Phl p 5 could be used as markers for potential risk of future asthma (27). We did not find the same association. In fact, neither sIgE-Phl p 1 nor sIgE-Phl p 5 were related to asthma. However, our patients with asthma presented not only higher levels of total IgE but also higher levels of sIgE against HDM, supporting the hypothesis that a more severe allergic respiratory disease is associated with higher levels of both total IgE (28, 29) and sIgE (30). However, and even though the study was performed in a population of patients with grass pollen allergy, our results led us to suspect that the weight of HDM sensitisation is what really mattered in our patients. This idea is supported by the result of the multivariate analysis, which demonstrated that the only variable relevant in asthmatic patients was HDM sensitisation, specifically sensitisation against *Dermatophagoides pteronyssinus*, the main allergen in our region (5-8, 20). Furthermore, the higher level of sIgE to *Dermatophagoides pteronyssinus,* the higher the risk of asthma. From a clinical standpoint, these results might be of importance, given dual sensitisation against grass pollen and *Dermatophagoides pteronyssinus* could help identify persons at risk of asthma in regions with similar levels of exposure to HDM. Nevertheless, a clinical problem arises when both sensitisations, grass pollen and HDM, coexist in the same patient. In an observational, Internet- and telephone-based prospective survey performed in Italy, France, and Spain of 313 patients with HDM allergy, 67% of the Spanish patients with HDM allergy reported co-sensitisation to grass pollen (8). The problem is that even 74% of patients allergic to HDM alone reported moderate seasonal variations in symptom scores, with peaks in spring and autumn, and these recalled seasonal changes could not be explained by concomitant, intermittent grass pollen allergy, but by the HDM sensitisation itself (8, 31, 32).

Conclusions

Taken together, these results suggest Phl p 1 is the most relevant allergen in patients with grass pollen allergy in the northwest of Spain, where profilin and polcalcin appear to be of low relevance. However, putting aside the sensitisation profile of patients with grass pollen allergy, HDM sensitisation is the most important variable that influences the clinical expression of respiratory symptoms.

Conflict of interests

The authors declare that they have no conflict of interests.

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Flour sensitization in a wooden door factory: what is the relationship?

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To the Editor,

Workplace exposures contribute substantially to asthma global burden, being related to 15-33% of adult asthma (1, 2). A wide variety of occupational substances have been associated to work-related asthma (WRA) (3). Over the last decades, significant changes occurred in workplaces with increasingly new agents introduced to work environment (3). Therefore, a complete occupational history of current and past jobs as well as hobbies is essential (2). Patients may not be aware of the exact work exposure, so all potential substances should be considered (1).
We report the case of a 23-year-old woman with no relevant medical history, referred to our department for recurrent episodes of dry cough, dyspnea, nasal congestion and sneezing, along with nasal and ocular itching in the past 12 months. The patient had been working in a wooden door factory for 3 years, operating a glue applier machine to unify wood veneers, being exposed to multiple substances without respiratory protection: wood dust, glue, paint vapors and thinners. Symptoms were exacerbated at workplace, especially one week a month when she prepared the

glue, which includes the addition of rye or wheat flour to several liquid products, as resins and hardeners. Symptoms improved on the rest of the month, vacations and weekends. She had no symptoms outside the workplace, tolerating cereal flour ingestion even with concomitant exercise. She also denied previous occupational exposure to flour (4). Improvement of symptoms was reported with inhaled budesonide/ formoterol 160/4.5 µg twice-daily and intranasal fluticasone 200 µg daily.
Spirometry at first visit revealed normal baseline lung function with a moderate hyperresponsiveness to methacholine bronchial provocation test (PC20: 0.36 mg/ml) (5). Occupational asthma (OA) was based on FEV1 determined after the work shift on the week she prepared the glue, performed at the hospital's pulmonary function laboratory according to ERS/ATS criteria (mean FEV1 2.6 L [89.7%]; mean increased FEV1 post-bronchodilation 540 cc [16%]), and 6 weeks after the last period in which she made this task (mean FEV1 3.2 L [108%]; mean increased FEV1 post-bronchodilation 60 cc [2%]).
Skin prick tests (SPT) with common inhalant and flours (Roxall-Aristegui, Bilbao, Spain) were positive to birch pollen and

to rye, wheat, barley, oats, and rice. Serum total IgE was 207 IKU/L. Serum specific IgE (ImmunoCAP, Thermofisher, Sweden) was elevated for rye (32.30 UK/L), wheat (9.10 UK/L) and barley (7.78 UK/L). Despite being sensitized to Tri a 14 (4.41 UK/L), a major allergen associated with baker's asthma, Tri a 19, commonly associated with wheat-dependent exercise-induced anaphylaxis (WDEIA), was found negative (0.01 UK/L) (6). She denied any symptom with ingestion of LTP containing foods. Patch-testing with Portuguese baseline, plastic and glues series, epoxy series (Chemotechnique Diagnostics™, Vellinge, Sweden) as well as wheat and rye flour (commercial flours without yeast diluted in isotonic saline solution) were negative (7).

The patient was diagnosed with OA with sensitization to rye, wheat, and barley. In this factory, cereal flour (rye and wheat) was added to increase the viscosity of urea-formaldehyde glue used in veneer panels. We recommended her immediate removal from glue preparation area as well as exposure to flours. Six months after relocation the patient became asymptomatic and lung function returned to normal, despite remaining in contact with different woods and other chemicals such as formaldehyde. She preserved her job in another workstation without flour exposure. The case was reported to the appropriate public health authorities, in accordance with national regulation, waiting for evaluation.

In summary, we report a case of OA with sensitization to cereal flours in a wood industry worker. The diagnosis of OA was based on bronchial symptoms and lung function improvement on days away from work and decline with labor exposure, in a patient without previous diagnosis of asthma. Although specific inhalation challenge is considered an important tool in OA diagnosis, a negative challenge in a worker with evidence of OA does not exclude the diagnosis (8). It was not performed in our case, since it is not available in our hospital.

Our first suspicion was an occupational sensitization due to resins, woods, hardeners, isocyanates or glue formaldehyde, the most commonly used materials in this industry. We therefore investigated the different chemicals and woods used by the patient in past and current jobs and hobbies, and the contact with cereal flours became distinctive. In some veneer panels factories, cereal flours are used to increase glue viscosity. This addition reduces the cost and improves the look of final product (9).

Rye and wheat were the known flours used in this factory, however, the patient was also sensitized to other cereals such as barley. This sensitization pattern could be explained by small amounts of these cereals contaminating the rye and wheat flours. Another hypothesis could be the cross-reactivity between wheat, rye, and barley due to homology among inhibitors subunits of these cereals (10).

Wheat is the main culprit for OA caused by flours, also known as baker's asthma (BA). Although not yet entirely characterized, wheat proteins are the most studied among all cereals. Salt-soluble proteins, namely α-amilase trypsin inhibitor family and non-specific lipid transfer protein Tri a 14, seems to be the main proteins

associated with BA and wheat food allergy. Tri a 19 (Ω-5 gliadin), a salt-insoluble protein, is commonly related with WDEIA (4, 6). Component-resolved diagnosis using Tri a 14 can help to clarify the sensitization pattern and route. In BA, Tri a 14 was characterized by Palacin *et al.* (6) as a major allergen, in contrast with Sanders *et al.* (10) who reported it as a minor allergen.

In this case, a finding of a positive Tri a 14 (4.41 UK/L) along with negative Tri a 19 supports the sensitization by occupational exposure and not a wheat seropositivity based on cross-reactivity with grass pollens (11). Since the patient denied previous work in bakery industry or other area including cereal flours, the sensitization seems to have happened at the wooden doors factory. To our knowledge, our case report joins three other cases of flour sensitization in wood industries described by López-Rico *et al.* in Spain (9). Nevertheless, strong data about flour sensitization in wood industry was not found. OA produced by flour is well-known in bakers and millers but this finding in wood industry workers is unexpected and reveals the importance of a detailed occupational history.

Conflict of interests

The authors declare that they have no conflict of interests.

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Speculations on red meat allergy due to α-Gal; its connection to coronary artery disease, suggested dietary guidance and allergy testing

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To the Editor,

in 2009, we reported a case-report of generalized allergic reaction during the performance of allergy tests to red meats (1). It was the case of a 59-year-old male, with a 10-year-long anamnesis of several anaphylactic episodes (urticaria-angioedema and asthma attacks) 2 hours after the consumption of mammalian meats. He was tolerating dairies and avian meat. His medical history was including seborrheic dermatitis, gastric ulcer, coronary artery disease (CAD) and symptoms of exercise-induced bronchospasm (1). Since all reactions were reported to happen after the ingestion of well-cooked meat we concluded that the culprit allergen was heat-stabile, without being able to specify it. Two more cases of allergy to red meat, males, 68-year-old and 52-year-old respectively, were referred to us last year, both confirmed with skin prick tests. The 68-year-old patient reported tolerating small quantities of cold cuts. They both had anamnesis of CAD; the 68-year-old patient had a stent placement (14 years ago), while the 52-year-old patient was under conservative medical treatment (for the last 6 years). At that time, CAD had been recently described as comorbidity to mammalian meat allergy and α-Gal allergen was inculpated (2). Patients' sensitization to α-Gal was later confirmed with specific IgE test (sIgE) against this allergen. We tried to contact the first case of meat allergy in order to prescribe the same test, but unfortunately we were informed that he had passed away due to myocardial infarction.

In the 2010s, tick bites were recognized as the main "sensitizer" to α-Gal, causing cross-allergic reactions to mammalian meat (3). Our patients are located in the rural area of the island of Euboea, Greece, engaged in outdoor activities and tick bites seem the most reasonable explanation of their sensitization. They confirmed anamnesis of tick bites but couldn't define the time. Three genera of *Ixodidae* family are the main ticks parasitizing humans in Greece; *Rhipicephalus*, *Ixodes* and *Hyalomma* (4). Al-

though not all tick bites cause IgE-sensitization to α-Gal, the above mentioned do (3, 5).

Alpha-Gal has been recognized as the culprit allergen for severe and fatal anaphylaxis to the mAb cetuximab, while case-reports have been published also for drugs like heparin, vaccines and anti-venom (3, 6). Although parenteral administration can cause immediate allergy, food allergy due to α-Gal is commonly expressed with a delay in symptom onset and is dose-unrelated; features also noticed in our cases (7-9). The pathophysiological mechanism differs when α-Gal is administered *via* the parenteral route than intake *via* the gastrointestinal system. α-Gal parenteral administration (*e.g.*, injection of cetuximab) triggers an acute IgE-mediated reaction, while a delayed allergy is observed when it enters through the digestive system.

The pathophysiological background of the "digestive" delay has been elucidated by an *in vitro* study, analyzing the transport of α-Gal through the intestinal epithelium (10). It was found that only the lipid-bound α-Gal is able to cross the intestinal epithelium, while protein-bound α-Gal was not detected in the basolateral media of enterocytes (10). Alpha-Gal contained in glycolipids is digested, absorbed, and enters the blood stream by the thoracic duct about 2 hours later, explaining the late-onset of allergic symptoms (3, 10). Furthermore, in α-Gal allergic patients, dairies may cause delayed onset of gastrointestinal symptoms over 2 hours (11).

There is a strong epidemiological connection between CAD and "α-Gal syndrome", a term used to describe different clinical allergies due to this allergen (12). This relationship has been confirmed by a study using intravascular ultrasound imaging in subjects undergoing cardiac catheterization (2). A mechanistic model has been proposed to clarify this connection, describing the delivery of α-Gal epitopes – connected to lipid particles – to mast cells within atherosclerotic plaques (12).

Due to the intraindividual tolerability to the culprit allergen, patients with α-Gal allergy exclude or reduce mammalian meat from their diet, but often consume tolerable quantities of products containing α-Gal. This can induce local mast cell degranulation leading to chronic mast cell activation and pro-inflammatory events contributing to the chronic inflammatory procedures of CAD pathogenesis (12). Our objection to this theory is that if mast cells play a pivotal role to this inflammation, red meat ingestion would cause a massive mast cell degranulation in atherosclerotic plaques so angina would be a common symptom of the deayed-type allergic reactions to red meat, resembling to Kounis Syndrome (13).

The hypothesis that small tolerable quantities cause the ongoing coronary inflammation *via* local mast cell degranulation is an emerging concern for us. Based on the knowledge that partici-

pation of chylomicrons and inflammation are common parameters of CAD and α-Gal sensitization, their exact immunological connection remains to be clarified. In order to avoid worsening of CAD by accumulation of lipoproteins containing α-Gal, we recommend the strict avoidance of all α-gal containing food, regardless the tolerance-level of each patient; dairies, gelatin and mammalian meat products should be avoided. Further large scale studies including metabolomic changes in such patients, can induce more clear evidence and conclusions.

Six prick-to-prick tests (fresh food) along to 3 SPT (HAL Allergy, The Netherlands) to red meats (beef, pork, mutton) had been used in our first case, leading to an unpredictable quantity of "fresh" allergen that penetrated skin, causing anaphylaxis. Prick-to-prick tests to a variety of fresh allergens of the same food group are performed in order to detect/exclude any differences in sensitization. In the case of mammalian meat products α-Gal appears to be present in all of them, but its concentration is higher in innards than in muscle meat (14).

Regarding the diagnostic procedures for red meat allergy, skin tests can set the diagnosis of allergy to red meat, but their low concentration of α-Gal may result in low sensitivity, as formerly reported (15, 16). On the contrary a blood assay for sIgE to α-Gal is more sensitive to confirm or set the diagnosis of α-Gal syndrome.

Skin tests with the use of commercially available SPT extracts were positive in all our cases. The precept from our initial case was that initially SPT with commercial extracts should be used and in the case they result positive, prick-to-prick should be omitted, in order to avoid the extremely rare – but possible – case of a systemic reaction. The use of prick-to-prick to different meat products are suggested as the next step to SPT, since they can offer additional information in cases of equivocal SPT (15, 16). Oral food challenge is considered the diagnostic gold standard for dubious cases, however in the case of red meat allergy the particularly delayed and potentially severe expected reactions suggest that this procedure can be carried out only to monitored inpatients.

Concluding, until larger epidemiologic, clinical and lab studies will clear the landscape of the connection between CAD, atopy and mammalian meat allergy (**table I**), it would be wise to advice and educate patients with α-Gal allergy for a strict avoidance of all α-Gal containing products and prescribe a regular cardiovascular check-up. The other way around, CAD patients should be probably checked for α-Gal sensitization in order to avoid accumulated inflammatory complications.

Conflict of interests

The authors declare that they have no conflict of interests.

Table I - *Unmet needs for α-Gal and the related CAD pathogenesis.*

Is the use of α-Gal-containing medications also connected to an increased risk of CAD?
What is the real impact of α-Gal ingested as food traces, on the pathogenesis of CAD in allergen-sensitized subjects with no red meat allergy?
Should the diet of a person sensitized to α-Gal be completely α-Gal-allergen-free?
If "lipid-bound α-Gal" is causing the chronic inflammation underlying CAD pathogenesis, is there the possibility that another food might also connect with CAD?
Should an α-Gal sIgE lab test be prescribed to all CAD patients?
Should α-Gal be mentioned in food labeling?
Is it possible to produce a commercial SPT extract of α-Gal? Can it be produced by red meat or ticks?

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